Premature Chromosome Condensation Studies in Human Leukemia.  
2. Proliferative Potential Changes After Induction Therapy for AML Patients

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Twenty-two patients with acute myelogenous leukemia (AML) were studied serially during remission induction therapy using the technique of premature chromosome condensation (PCC). Mononuclear cells from bone marrow aspirations were fused with mitotic Chinese hamster ovary cells, and the PCC were scored for fraction of cells in G1, S, and G2. The proliferative potential index PPI, or fraction of the G1 cells in late G1, and degree of chromosome damage were also determined. These results were compared to the changes in bone marrow morphology and blast counts during therapy and correlated with the clinical outcome. Patients who responded to chemotherapy generally exhibited an initial drop in PPI concomitant with chromosome damage. This was followed by a rise in the PPI and fraction of S-phase cells prior to clinical evidence of bone marrow regeneration. As the bone marrow and blood continued to regenerate to normal levels, the PPI value decreased to intermediate levels (less than 35%). In contrast, patients who did not achieve complete remission were observed to exhibit no detectable PCC changes or continued high PPI values after regeneration. High blast counts in the marrow during the regenerative phase accompanied by low PPI values were correlated with a favorable prognosis, since all patients with such a pattern achieved complete remission in that course or the subsequent course of therapy. The PCC technique, therefore, is useful in monitoring response to remission induction therapy in AML patients and can complement morphological and cytogenetic parameters. This ability might be especially useful during the regenerative period when normal and leukemic regeneration is difficult to distinguish by classical techniques.

The effectiveness of remission induction therapy in patients with acute leukemia is commonly monitored by changes in the blood and bone marrow cellularity and morphology. These parameters alone are insufficient to predict whether the patient will achieve a lasting remission or only a transient response. The purpose of this study was to determine whether the phenomenon of premature chromosome condensation (PCC) might be useful in the prediction of response during remission induction therapy and as an indicator for remission duration in patients with acute myelogenous (AML) or acute monomyelocytic leukemia (AMML).

When interphase cells and mitotic cells are fused together using Sendai virus, the interphase chromatin is forced to condense into discrete units that are known as prematurely condensed chromosomes or PCC. Early studies in our laboratory have indicated that the phenomenon of premature chromosome condensation can be a useful tool in the study of the proliferative and cytogenetic characteristics of both normal and tumor cell populations. The morphology of the PCC reflects the cell-cycle phase just prior to fusion (G1, S, G2, or M). In addition, the degree of chromosome condensation of the PCC reflects the position of the cell within each phase. For example, early G1 cells give rise to condensed G1 PCC, while late G1 cells give rise to highly extended G1 PCC. The PCC also can be used to determine chromosome number of the cells in G1 and G2 phases. Thus, in one fusion procedure, one can obtain both a cytokinetic and cytogenetic analysis of cell populations.

Early studies have also indicated that normal and malignant cell populations can be distinguished by the PCC technique. Normal cells in a resting phase appear to accumulate in early G1 phase, while transformed cell populations in plateau conditions accumulate in late G1 phase. This same phenomenon holds true in the case of human bone marrow populations and solid tumors. Bone marrow cells obtained from patients with untreated acute leukemia generally exhibit an increased fraction of G1 cells in late G1 phase (high proliferation potential index or PPI) when compared to specimens obtained from subjects with normal bone marrows. This accumulation in late G1 phase is not correlated with the fraction of cells in S phase.

In a preliminary communication, we used the PCC technique to study several leukemic patients at different stages of their disease and therapy. While single patients were not serially monitored at that time, the data for the groups as a whole suggested that at the end of successful induction combination chemotherapy, the PPI drops to values intermediate between normal (average, 12%) and initial acute leukemic...
levels (average, 35%-40%). In the case of progressive disease, however, the PPI remains high (> 35%). It was of interest, therefore, to determine whether early changes in the PCC characteristics after therapy could predict the subsequent clinical course, i.e., eventual response or progression of disease. To this end, 22 patients with acute myeloid leukemia were followed through remission induction therapy using the PCC technique. Reproducible kinetic and cytogenetic trends were observed to accompany therapeutic responses that were not observed when there was little or no response. In certain cases, the PCC technique provided information uniquely predictive for the future course of the disease.

MATERIALS AND METHODS

Patient Selection and Characteristics

Twenty-two patients with documented acute myelogenous leukemia were chosen for sequential bone marrow studies during remission induction therapy. Nineteen of these patients had AML, while three patients exhibited AMML. Twenty-one of the patients were previously untreated for their disease, while the other patient previously received vincristine and prednisone. The median age of the patients was 61 yr, with range from 20 to 74 yr. The average bone marrow blast percentage was 83.3% (range, 48.8%-96.4%), and the average leukemic blast cell infiltrate (product of cellularity and the blast percentage) in the bone marrow was 72.1% (range, 48.8%-87.2%).

Karyotype studies of bone marrow specimens performed by the cytogenetics laboratory showed 9 patients with all diploid cells (NN), 5 patients with a mixture of abnormal and normal karyotypes (AN), 3 patients with all abnormal metaphases (AA), and 2 patients with insufficient numbers of metaphases for analysis. Cytogenetic analyses was not performed on the remaining two patients. All patients were treated at the University of Texas System Cancer Center M.D. Anderson Hospital and Tumor Institute during the period 1976-1977, and all patients received combination chemotherapy, including an anthracycline (adriamycin or rubidizone), vincristine, cytosine arabinoside, and prednisone (Ad-OAP and ROAP, respectively).

Bone Marrow Cell Preparation

Bone marrow specimens were obtained and processed for cell fusion as previously described. Briefly, mononuclear bone marrow cells were obtained by the Ficoll-Hypaque centrifugation technique. Where two bands of cells were observed in the gradient, both bands were combined for these studies. The mononuclear bone marrow cells were then washed and fused with mitotic Chinese hamster ovary (CHO) cells (greater than 95% pure, obtained by selective detachment of Colcemid-arrested cells) using Sendai virus. At the completion of cell fusion and the induction of PCC, the fusion mixture was treated with hypotonic 0.075 M KCl for 10 min, fixed in methanol-glacial acetic acid (3:1, v/v), and dropped on wet microscope slides. These chromosome preparations were then stained with Giemsa (Fisher).

Scoring of Slides

For each fusion between patient bone marrow cells and mitotic CHO cells, 100 PCC were located first at low power and then scored at high power with the light microscope. Each PCC was scored for its position in the cell cycle (G1, S, or G2) according to PCC morphology and analyzed for visible evidence of chromosome damage (gaps, breaks, exchanges, stickiness, etc.). Each G1 PCC observed was graded for its degree of condensation on an arbitrarily defined scale of 1-6, with a value of 6 representing the most highly extended G1 PCC. For the 100 PCC scored for each slide, the cell-cycle distribution for the bone marrow populations was determined by the fraction of PCC of G1, S, and G2 types. To quantitate the distribution of G1 cells within G1 phase, and ratio of the number of highly extended G1 PCC (classes 4, 5, and 6) to the total number of G1 PCC observed was determined; this value is defined as the proliferative potential index (PPI). In essence, the PPI represents the fraction of G1 cells in late G1 phase. Chromosome damage to the bone marrow as a whole was graded on a scale of 0-4, with 4 representing a population of cells exhibiting extensive chromosome damage visible in the PCC. To avoid bias, bone marrow PCC slide preparations were coded and scored without knowledge of the patient's disease status or stage of therapy. Contamination by CHO cells was not a problem, since the CHO mitotic populations were routinely nearly pure.

RESULTS

Bone marrow aspirations were evaluated by the PCC technique prior to initiation of therapy, periodically after the first course of chemotherapy until bone marrow recovery, and at the initiation of the second course of therapy. In some patients, aspirations were...
Table 1. Relationship Between Pretreatment PPI Values and Clinical Outcome

<table>
<thead>
<tr>
<th>Initial PPI</th>
<th>No. Patients</th>
<th>CR</th>
<th>PR or Failure</th>
<th>Early Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 35%</td>
<td>11</td>
<td>9</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Over 35%</td>
<td>11</td>
<td>8</td>
<td>2</td>
<td>1</td>
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also periodically evaluated after the second course of therapy. Of the 22 patients analyzed here, 17 achieved complete remission, 3 had either a transient or no response, and 2 patients died during induction therapy.

The pretreatment PCC characteristics for this group of patients is shown in Fig. 1. The average pretreatment PPI for this group was 37.4%, with a range of 12.0%–74.5%. The average fraction of S PCC was 6.7% with a range from 0%–14%. Little or no chromosome damage was observed in the bone marrow cells prior to the initiation of therapy. Table 1 illustrates the relationship between the high and low pretreatment PPI values and the subsequent response to induction therapy. In this small group of patients, pretreatment PPI values did not predict or correlate with eventual clinical response or survival.

The 9 patients with all diploid metaphases (NN) all achieved complete remission and had a median survival of more than 22 mo (range, 12–> 29 mo). Four of the five patients with a mixture of aneuploid and diploid metaphases in their marrow achieved complete remission and had a median survival of 11 mo (range, 7–>42 mo). Two of the three patients with all aneuploid karyotypes achieved complete remission, but all had short survival periods (0, 5, 14 mo). One of the two patients with insufficient numbers of metaphases achieved complete remission and survived 14.5 mo, while the other failed therapy and survived only 2 mo.

After the initiation of chemotherapy, the PCC characteristics of the bone marrow cells were observed to undergo many changes with respect to chromosome damage, PPI, and the fraction of cells in S phase. However, repeating patterns of PCC changes were observed in groups of patients who responded similarly. These typical patterns will be described in this section.

Early Changes (During Induction Therapy)

One patient was studied very early during the course of induction therapy. The patient presented with a 100% cellular marrow with 86% blasts and a peripheral white blood cell count of 106,000/μl with 92% blasts. The pretreatment bone marrow PPI and percent S-phase cells were 24.1% and 9%, respectively, with 4% of cells in G2 phases. The cytogenetic pattern is noteworthy, since this patient exhibited a ring chromosome in both G1 and G2 PCC (Fig. 2A and B), and all metaphase figures exhibited aneuploidy (44–46 chromosomes) with evidence of chromosome instability and damage. The patient was then started on ROAP remission induction therapy. By day 3 of therapy, the PPI dropped to 11.3%, the fraction of S PCC was 10%, and the fraction of cells in G2 phase had risen to 19%. Extensive chromosome damage was observed in the PCC (Fig. 2C and D: i.e., 15 of 19 G2 PCC observed showed chromosome damage). Of particular note, many chromatid-type exchanges (typical of anthracycline-induced damage were observed. While the marrow blast percentage had dropped to 57.8% by day 3, the patient developed an infection and died on day 5 despite antibiotic therapy.

Patients Achieving Complete Remission

In 17 of 22 patients who eventually achieved complete remission, reproducible changes in the PCC characteristics of serial bone marrow aspirations were observed. Figure 3 illustrates the typical PCC pattern changes for such a patient during the remission induction period; we have plotted the PPI, fraction of cells in S phase, degree of chromosome damage, and the bone marrow blast counts as a function of time. Prior to the initiation of therapy, this female patient showed a 90%
cellular bone marrow with 82% blasts (peroxidase positive) and 2.8% promelocytes. Cytogenetic studies showed all diploid metaphases. Peripheral blood analysis showed 8000/μl white blood cells with 70% blasts, 10% polys, and 20% lymphocytes. A PCC evaluation of the pretreatment bone marrow showed a PPI of 18.9% with 5% cells in S phase. No chromosome damage was apparent in the PCC. The patient was then treated in the protected environment with a standard dose of Ad-OAP therapy.

The first posttreatment bone marrow to be analyzed was obtained on day 8 of the first course of therapy. At this time, the PPI and percent S-phase PCC had not changed significantly; however, chromosome damage was evident in the bone marrow. The bone marrow blast count had dropped to 4.7% with 35% cellularity. At this time, most of the chromosome damage observed was in the form of open chromatid gaps and breaks with little or no exchanges; this type of chromosome breakage pattern is typical of cytosine arabinoside-induced damage.

Subsequently, the bone marrow became hypocellular. Just prior to regeneration of the bone marrow, the PPI and percent S PCC increased markedly. As the bone marrow continued to regenerate to normal levels, the PPI decreased, while the fraction of cells in S phase remained at intermediate levels. Significant levels of chromosome damage continued to be observed throughout the first course. By day 31 of the first course of therapy, the patient was clinically in complete remission, and the PPI was low (9%) with 18% of the cells in S phase. With subsequent courses of consolidation and maintenance therapy, the PPI was observed to remain under 35%, the percent S PCC remained at intermediate levels, and the chromosome damage due to therapy continued to be evident in the bone marrow. This patient remained in complete remission for 13 mo.

Four of the 10 patients with high initial PPI values (>35%) achieved complete remissions in the first course of therapy. The pattern observed in these patients was similar to that described above, i.e., a regenerative phase accompanied by high PPI and S-phase values followed by a drop in the PPI values. However, in two of the patients, the PPI values began to increase just prior to the second course of therapy (up to 36% and 41%, respectively). Nevertheless, by the end of the second course of therapy, the PPI values had dropped to 30% and 11%, respectively, with significant fraction of cells in S phase and chromosome damage evident in the PCC.

One of the four responding patients with a high initial PPI value showed a PCC pattern during remission induction therapy that proved to be quite useful in his treatment. This male patient presented with a 90% cellular marrow with 89% blasts and a white cell count of 31,000/μl and 72% blasts. Cytogenetic analysis showed only diploid metaphases. As shown in Fig. 4, pretreatment PCC evaluation showed a high PPI and low fraction of S-phase cells. The patient was then treated with Ad-OAP and the PPI dropped, the fraction of cells in S-phase increased, and chromosome damage was evident in the PCC. By the end of the first course of therapy, the patient was considered in complete remission. On day 17 of the second course of ROAP therapy, however, the blast percentage in the bone marrow had risen to 54.5%. At this time there was concern whether this represented relapsing leukemia or normal regeneration. Since the presenting leukemic clone was diploid, cytogenetic analysis could
At the end of the first course of therapy, all 6 patients still exhibited significant percentages of blasts in the bone marrow, and it was difficult, by morphological standards, to predict whether these patients would ever achieve complete remission (CR). Five of the six patients, however, exhibited relatively low PPI values with evidence of chromosome damage at the end of the first course, and all five patients achieved CR in the next course of therapy. The sixth patient, however, showed a high PPI (53%) and 68% blasts in the marrow at the end of the first course of therapy. By the end of the second course, the patient had achieved complete remission and the PPI had dropped to 4.2% with 26% S-phase cells and evident chromosome damage. The end of the third course of therapy, however, was marked by a high PPI (46%), and the PPI remained high over the next 5 mo, at which time the patient began to show clinical evidence of relapse. Thus, a continued high PPI was suggestive of progression of disease.

**Patient Achieving a Partial Remission**

One of the five patients not achieving a complete remission did achieve a partial remission. This male patient presented with a 95.4% cellular marrow with 91.6% blasts and a peripheral blood white cell count of 6900/μl with 14% blasts. Cytogenetic analysis showed 65% hypodiploidy in the marrow.

PCC evaluation of the presenting bone marrow showed a PPI of 55.3% with 2.1% cells in S phase. The patient was begun on ROAP therapy. As shown in Fig. 5, during the first course of therapy, the PPI remained high, the S fraction low, little chromosome damage was observed, and there was little effect on the bone marrow blast percentage. By the end of the second course of therapy, the blast percentage remained high but the PPI dropped to 32.3%. This drop in PPI was followed by a drop in blast percentage during the third course of therapy and a rise in the fraction of cells in S phase and the amount of chromosome damage. At the end of course 3, the patient was considered to be in partial remission. However, the PPI rose again to a high value (54.8%) by the end of the third course. At the end of the fourth course, the blast percentage dropped to under 10%, yet the PPI remained above 35%, predictive of progressive disease. During the fifth course, the PPI remained high, the S fraction fell to low values, and little chromosome damage was apparent. The patient's disease then clinically progressed and he was placed on neocarzinostatin therapy.

**Patients Not Responding**

Four of the original 22 patients in this study did not achieve complete or partial remissions. Two of these...
patients suffered early deaths. The first of these was described earlier, and the second showed clinical (drop in marrow blast percentage) and PCC evidence of response (drop in PPI, rise in S, and chromosome damage) but developed respiratory arrest and died on day 13 of the second course of therapy. The third of the four nonresponding patients showed continued high PPI values with little chromosome damage despite therapy and showed little response in the bone marrow. After three courses of Ad-OAP therapy with little evidence of response, the patient was placed on 5-azacytidine therapy.

The fourth nonresponding patient presented with 95% cellular marrow and 93.8% unclassified cells (peroxidase positive, PAS negative, NS esterase negative, insufficient number of metaphases for cytogenetic analysis) and a white blood cell count of 24,200/μl with 85% undifferentiated cells. The patient was initially treated with antibiotics for a throat infection and then initiated on Ad-OAP remission induction therapy. As shown in Fig. 6, little change was observed in any of the PCC characteristics during the first course of therapy. After the next course, chromosome damage was evident, the fraction of cells in S phase increased, and the blast percentage dropped slightly. However, by the end of the second course, the PPI jumped to high values, the S fraction dropped to lower values, and no chromosome damage was evident. The patient received a third course of Ad-OAP with little evident response and therapy was changed to 5-azacytidine.

DISCUSSION

These studies were initiated to determine whether the technique of premature chromosome condensation might be useful both in understanding the regenerative phase after remission induction therapy and in the early prediction of response after remission induction.
Previously reported studies had shown that bone marrow populations from untreated leukemia patients generally show higher proliferative potential indices (PPI) than do normal bone marrow populations, while bone marrow cells from leukemic patients in complete remission show intermediate PPI values. On the other hand, patients with progressive disease had shown continued high PPI values. However, in the early studies we had sampled different patients in different stages of disease. In this study we serially followed several patients through remission induction therapy and determined the relationship between normal and leukemic regeneration as viewed by their PCC characteristics.

These results show that, in general, response to therapy is correlated with an initial drop in the PPI accompanied by chromosome damage, followed by a rise in the PPI and fraction of cells in S phase during early regeneration. As regeneration continues, the PPI falls to intermediate values (under 35%), while the fraction of S-phase cells remains greater than 10%. By the end of a successful course of therapy, residual chromosome damage is still evident in the PCC. Failure to achieve complete remission is accompanied by either no change in PCC characteristics or continued high PPI values.

Table 2 illustrates the clinical situations in which the PCC technique might be extremely helpful in determining prognosis. When patients were clinically not in complete remission at the end of a course of therapy, a low PPI value was prognostic of eventual complete remission (5/6), while a high PPI was prognostic of nonresponse (¼ achieved CR). The one patient with a low PPI had presented with a low PPI and little PCC change was observed with therapy (Fig. 6). The one patient who eventually achieved a CR despite a high PPI only showed a transient response and quickly relapsed.

The third line of Table 2 illustrates another problematic clinical situation. In these cases, significant numbers of blasts are still present in the bone marrow during the regenerative phase, and it is difficult to distinguish normal from leukemic regeneration. In this study, all seven patients who exhibited low PPI values during this type of regenerative phase achieved complete remission. As will be reported in more detail in other publications, similar patterns of regeneration have been observed both in other leukemic patients and in the bone marrows of solid tumor patients recovering from intense combination chemotherapy and bone marrow transplantation. The converse situation (blasts present with high PPI) cannot be compared, since most patients exhibit a high PPI early in regeneration.

The above results suggest that complete remission is associated with a decrease in PPI values while progression is associated with high PPI values. However, as shown in the last line of Table 2, four patients were shown to have high PPI values despite clinical evidence of complete remission. Two of these patients showed only a short duration of remission. One of the remaining two patients was still in the regenerative phase after therapy, since there were 47.9% erythroid elements in the marrow at the time of sampling. Both responding patients showed decreased PPI values by the end of the next course of therapy and continued in complete remission. These results emphasize the need for serial monitoring of patients for full prognostic evaluation.

It is interesting to note that patients with all diploid metaphases (NN) survived longer than did patients with a mixture of abnormal and diploid metaphases. (>22 versus 11 mo median survival). Patients with all aneuploid metaphases or insufficient numbers of metaphases showed very short survival periods (median, 5 mo). While the number of patients reported here is small, these results support the observation of several other laboratories that total aneuploidy in the bone marrow is associated with a poor prognosis. However, two of the three patients exhibiting 100% aneuploid bone marrow metaphases did achieve complete remission. The PCC technique might be useful in the early detection of relapse in such patients by both detecting residual aneuploid cells during remission and by detecting abnormal proliferation characteristics.

The observations reported here also have biologic implications. For the majority of responding patients, even as the blast count decreased, bone marrow regeneration was preceded or accompanied by a sharp rise in both the PPI and fraction of cells in S phase. This finding is reminiscent of the early proliferative
changes observed in normal peripheral blood lymphocytes stimulated to divide in vitro with phytohemagglutinin. This dramatic rise in the proliferative potential also corresponds in time to the rise in circulating colony-stimulating activity reported for serum obtained from leukemic patients after therapy. The types of chromosome damage observed during and after therapy may reveal the contributions of the various components of the therapy. The earliest damage observed was in the form of gaps, breaks, exchanges, and sticky chromosomes. Anthracyclines have been shown in vitro to cause this pattern of chromosome damage for cells in all phases of the cell cycle. Damage observed later in the course of therapy was in the form of chromosome breaks and gaps but little or no exchanges. This damage is reminiscent of chromosome damage produced by cytosine arabinoside (AraC) and requires the cells to be exposed to AraC close to or during S phase. These observations, coupled with our in vitro observations that exchanges, once formed, cannot be repaired, suggest that the role of anthracycline in induction therapy is to induce a significant cell kill (resulting in loss of cells with chromosome exchanges from the bone marrow). This would then allow leukemic cell recruitment from the resting phase. These proliferating cells are then attacked by cytosine arabinoside action.

The morphology of the PCC in G1 phase in the patients studied suggests the leukemic cell chromatin is more highly extended than that of normal resting cells. The property of extended chromatin in leukemia cells might also extend to mitotic cells where one often sees a fuzzy appearance in AML patient chromosomes. In fact, neoplastic cells in general have been found to have increased DNA helix openings even at mitosis. Since intercalating agents such as anthracyclines would be expected to bind to a greater degree to extended chromatin, this may provide for a differential sensitivity of leukemic cells to anthracycline treatment when compared to normal cells.

The ability of the PCC technique to predict for response during the regenerative period after therapy is limited by the fact that the PPI is observed to increase both during early regeneration of normal marrow and also during leukemic regeneration. While normal bone marrow regeneration was often found to be characterized by a rise in the S-phase fraction and significant chromosome damage, this was not always the case. In patients with distinct cytogenetic markers for leukemic cells, one future possibility would be to analyze the PCC of bone marrow cells for the presence of cells exhibiting the cytogenetic marker.

In conclusion, the phenomenon of premature chromosome condensation appears to be a useful tool with which to monitor the pattern of response of patients with acute myeloid leukemia to remission induction therapy. Patients responding to therapy showed reproducible patterns in their PCC characteristics during the course of therapy. These patterns were not observed in those patients not responding or only partially responding to therapy.

The PCC technique was especially helpful in predicting response both during the regenerative phase in some patients and in cases where multiple courses were required to achieve complete remission. Future study of patients during the initial days of induction therapy might allow for a quick correlation between initial induction of chromosome damage and eventual response. This type of knowledge might allow for individual tailoring of therapy for each patient according to the patient’s in vivo determined sensitivity to the therapy regimen. Such future studies will hopefully also give insight into proliferative controls in the leukemic and normal bone marrow.

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