RAPID COMMUNICATION

Proof of Differentiative Mode of Action of All-Trans Retinoic Acid in Acute Promyelocytic Leukemia Using X-Linked Clonal Analysis

Suzanne Elliott, Kerry Taylor, Susan White, Robyn Rodwell, Paula Marlton, Damien Meagher, James Wiley, Debra Taylor, Sue Wright, and Peter Timms

Using X-linked clonal analysis, mechanism of action of all-trans retinoic acid (ATRA) was sought in a 16-year-old female with relapsed clonally evolved acute promyelocytic leukemia (APL), who achieved complete remission. On ATRA, metamorphosis of peripheral blood leukemic promyelocytes to mature neutrophils was observed, despite the persistence of t(15;17) in 100% of bone marrow metaphases. DNA was extracted from fractionated serial blood specimens, collected at diagnosis, in first complete remission (CR), relapse, and during ATRA treatment. Using a phosphoglycerokinase (PGK) probe, the patient was heterozygous for both BglI and BstXI PGK polymorphisms. Methylation analysis showed monoclonal leukemic promyelocytes with a polyclonal first CR achieved by standard chemotherapy. Subsequent examination, in relapse, of granulocytes appearing during ATRA treatment showed these to be monoclonal, proving these were derived from the neoplastic clone. The X-linked clonal analysis methodology has provided in vivo evidence of cellular differentiation as the mechanism of action of ATRA. Parallel studies of cytogenetic and clonal analysis showed a regression of the t(15;17) cytogenetic abnormality and return of a polyclonal PGK methylation pattern in 5 weeks, indicating a repopulation of marrow by normal stem cells. As standard cytogenetic techniques are inappropriate for nondividing cells, X-linked clonal analysis provides a marker system to allow insight into mechanism of drug action in malignant hematologic disease.

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PATIENTS AND METHODS

Patient history. A 15-year-old female presented with confusion and fever. Laboratory studies disclosed pancytopenia (hemoglobin [Hb] 48 g/L, white blood cells [WBC] 0.9 x 10^9/L, platelets 25 x 10^9/L) with abnormal circulating promyelocytes and accompanying coagulopathy (prothrombin time [PT] 37.4 seconds, activated partial thromboplastin time [APTT] 62.6 seconds, fibrinogen 0.9 g/L, D-Dimer > 32 mg/L). APL was diagnosed on bone marrow (BM) morphology and the standard cytogenetic translocation, t(15;17), demonstrated in 100% of BM metaphases.

Induction chemotherapy with Cytarabine (100 mg/m^2/d) by continuous infusion for 7 days, Daunorubicin (50 mg/m^2 intravenously [IV]) on days 1, 2, and 3, and Etoposide (75 g/m^2 IV) on days 1 through 7, was commenced. Postinduction marrow examinations showed CR without a phase of marrow hypocellularity. This was followed by consolidation and maintenance therapy.

The patient relapsed 1 year later while on maintenance treatment. Cytogenetics analysis confirmed the recurrence of the t(15;17) clone present alone in 30% of metaphases. A coexistent abnormality, 46,XX,t(15;17)(q22q12),-2, +der(2)t(2;?), was present in 70% of cells.

Initial efforts to obtain ATRA for reinduction proved fruitless and the patient was commenced on high-dose Cytarabine (3 g/m^2 on days 1 through 5) and Amsacrine (200 mg/m^2 on days 1 through 3). By day 13 of this therapy, leukemic progression was noted with reappearance of circulating promyelocytes and recrudescence of coagulopathy (Table 1). ATRA, obtained from Prof M.E. Huang (Shanghai, China), was commenced (day 0) at 45 mg/m^2/d. The coagulopathy resolved within the first week. Hydroxyurea was

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introduced at 2 g per day for 3 days on day 5 to moderate hyperleucocytosis. The patient entered complete cytogenetic remission and subsequently underwent allogeneic BM transplant from an HLA-matched sibling and is well and free of leukemia 7 months later.

**Cytogenetics.** Cytogenetic studies were performed on either BM or peripheral blood (PB). Cultures were set up without mitogens for overnight incubation and for 48-hour fluorodeoxyuridine with an immediate decrease in WBC noted. cant maturation of the myeloid cells was observed.

**DNA analysis and probes.** Restriction enzyme Bgl I was used to assess heterozygosity and, thus, interpretability for clonal analysis.

**Material studied.** Serial PB specimens were separated into polymorphonuclear (PMN) and mononuclear (MN) fractions by Ficoll-Hypaque centrifugation. Fraction purity was assessed by Giemsa staining, with granulocytes constituting greater than 90% of cells in the PMN fraction. High molecular weight DNA was extracted from the fractions by methods previously described.

**RESULTS**

**Clinical response.** Response to ATRA was rapid with a steady decrease in PB promyelocytes, increase in mature neutrophils, and improvement in coagulopathy (Table 1 and Fig 1). Hyperleucocytosis was easily managed with hydroxyurea with an immediate decrease in WBC noted.

**Morphology.** Within 1 week of ATRA treatment, the promyelocyte numbers reduced with the appearance of transitional "myeloid" cells and eventually mature granulocytes (Fig 1). Transitional cells had dysmorphic features of atypical nuclei and granule development. BM performed at day 7 showed persistent hypercellularity, although significant maturation of the myeloid cells was observed.

**Cytogenetics.** Initial BM cytogenetics at first presentation identified the standard t(15;17)(q22;q12) in 100% of cells. A complete cytogenetic remission was obtained post-chemotherapy.

On relapse, cytogenetics disclosed two abnormal cell lines, the standard 46,XX,t(15;17)(q22;q12), and a second, 46,XX,t(15;17)(q22;q12), -2,+der(2)t(2;?)(p25;?). Cytogenetically, 15% of the 20 metaphases demonstrated the standard translocation, and 85% showed the evolved clone at day 0 on ATRA (Table 2). At 1 week post-ATRA treatment, only the evolved clone was present from eight metaphases. Normal diploid metaphases appeared at day 14, with three cell lines present, the normal predominant, at day 28. Complete cytogenetic remission was apparent by day 35.

**Molecular.** The restriction fragment length polymorphism (RFLP) with Bgl I enzyme was identified in the patients DNA showing two bands, 12 and 5 kb, with the X-linked phosphoglycerokinase (PGK) probe (results not shown). The patient’s DNA was also heterozygous for the RFLP restriction enzyme, Bst XI, which has strong linkage (RFLP) with

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**Table 1. Serial Laboratory Data on APL Patient at Presentation, Relapse, and Post-ATRA Treatment**

<table>
<thead>
<tr>
<th>Test</th>
<th>Initial Presentation</th>
<th>Relapse Day-13</th>
<th>ATRA Day 0</th>
<th>ATRA Day 7</th>
<th>ATRA Day 35</th>
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<tr>
<td>Hb (g/dL)</td>
<td>4.8</td>
<td>12.5</td>
<td>9.8</td>
<td>9.6</td>
<td>10.5</td>
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<td>WBC (x10⁹/L)</td>
<td>0.9</td>
<td>3.5</td>
<td>7.6</td>
<td>20.0</td>
<td>8.2</td>
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<tr>
<td>AGC (x10⁹/L)</td>
<td>0.1</td>
<td>1.0</td>
<td>0</td>
<td>6.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Platelets (&gt;10⁹/L)</td>
<td>25</td>
<td>271</td>
<td>25</td>
<td>59</td>
<td>530</td>
</tr>
<tr>
<td>PT (s)</td>
<td>37.4</td>
<td>15.1</td>
<td>23.0</td>
<td>15.1</td>
<td>15.2</td>
</tr>
<tr>
<td>APTT (s)</td>
<td>62.6</td>
<td>32.8</td>
<td>46.3</td>
<td>39.3</td>
<td>33.0</td>
</tr>
<tr>
<td>FIB (g/dL)</td>
<td>0.9</td>
<td>2.9</td>
<td>2.4</td>
<td>2.5</td>
<td>2.4</td>
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<tr>
<td>D-Dimer (mg/mL)</td>
<td>&lt;32</td>
<td>0.5</td>
<td>64</td>
<td>&lt;0.5</td>
<td>NT</td>
</tr>
</tbody>
</table>

Abbreviations: AGC, absolute granulocyte count; NT, not tested; FIB, fibrinogen.

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**Table 2. Cytogenetic Findings in the BM of Patient During ATRA Treatment**

<table>
<thead>
<tr>
<th>Date</th>
<th>No. of Metaphases</th>
<th>Cytogenetic Cell Lines (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>46XX</td>
<td>t(15;17)+</td>
</tr>
<tr>
<td>Relapse</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>ATRA day 0</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td>ATRA day 7</td>
<td>8</td>
<td>100</td>
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<tr>
<td>ATRA day 14</td>
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<td>15</td>
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<tr>
<td>ATRA day 21</td>
<td>20</td>
<td>85</td>
</tr>
<tr>
<td>ATRA day 35</td>
<td>20</td>
<td>100</td>
</tr>
</tbody>
</table>

ISCN 1985.**

*46,XX,t(15;17)(q22;q12).
†46,XX,t(15;17)(q22;q12), -2,+der(2)(2;?)p25;?.

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Fig 1. Myeloid cell counts and corresponding cytogenetics during treatment in APL patient with relapse. Abbreviations: 15/17, t(15;17)(q22;q12); m-AMSA, amasacrine; HIDAC, high-dose Ara-C; PB AGC, PB absolute granulocyte count (-----); PB Promyelocytes, PB promyelocytes (---).
two X chromosomes. Subsequent methylation sensitive restriction enzyme (MSRE) digestion with Hpa II, identified a polyclonal pattern, with a 50% reduction in intensity of both bands (Fig 2, lane 1b). DNA extracted from the initial abnormal promyelocytes containing the unique translocation, t(15;17)(q22,q12), showed a total loss of a single band after MSRE digestion (Fig 2, lane 2b). From the X-linked methylation methodology, this confirms that the X-chromosome defined by the 0.9-kb band was entirely in the active form, proving these abnormal cells to be monoclonal. Relapsed promyelocytes demonstrating the additional abnormal −2,+der(2)(t;2)(p25;) clone to the standard 46,XX,t(15;17) translocation had the same pattern as the original abnormal promyelocytes, indicating that these were again monoclonal (results not shown). DNA extracted from the PMN fraction 7 days after ATRA treatment (D7), also displayed a single band after MSRE digestion, confirming a monoclonal pattern identical to that of the abnormal promyelocytes (Fig 2, lane 3b). The PGK analysis of the granulocytes at day 35 of ATRA treatment showed a polyclonal pattern with the presence of both bands (1.05 and 0.9 kb) after MSRE digestion (Fig 2, lane 4b). Hematologic and cytogenetic remission correlated with a return to a polyclonal methylation pattern.

**DISCUSSION**

ATRA is a highly effective single agent in relapsed or resistant acute promyelocytic leukemia. Original in vitro studies demonstrated that the retinoids could induce differentiation on patients’ abnormal promyelocytes and human cell lines. As conventional karyotypic analysis is restricted to dividing cells, this technique is not applicable to examine emerging neutrophils. Various methodologies have been used to evaluate the mechanism of action of ATRA on the leukemic cells and the nature of the resultant mature cells that appear after therapy.

Morphologically, the appearance of maturing neutrophils with Auer rods has been identified as a marker of differentiation of the abnormal immature cell. This asynchronous abnormal maturation is not found in all neutrophils and, as Tallman et al noted, is suggestive of, but does not definitively prove, the differentiative process. Myeloid cells from our patient appearing after ATRA treatment demonstrated dyshemopoietic features of nuclei shape and cytoplasmic granulation.

Warrell et al identified dual monoclonal surface markers of early (CD33) and late (CD16) myeloid specificity on the differentiated “intermediate” cells. Although, BM samples containing developing myeloid cells would contain cells with dual transitional surface markers, this finding in PB constitutes a significant finding. The specialized cytogenetic procedures of premature chromosome condensation and fluorescent-in situ-hybridization (FISH) were used by Warrell et al to identify the t(15;17) translocation in mature granulocytes from APL patients treated with ATRA. The limitation of this technique lies with the complex methodology and maintenance of Chinese hamster ovary cells.

X-linked clonal analysis provides an alternative methodology to investigate clonal origin of mature cells that appear after therapy. Analysis of the leukemic promyelocytes at presentation identified a monoclonal pattern consistent with a single cell origin. This result highlights the use of this extrinsic methodology to confirm monoclonality of the cells that already have an intrinsic cytogenetic clonal marker.

Analysis of granulocytes collected at 1 and 3 months postinitial induction chemotherapy demonstrated a polyclonal pattern in the presence of a hypercellular marrow. Differentiation has been postulated as the mechanism of hypercellular remission in APL and, indeed, monoclonal granulopoietic remissions have been previously noted in this disease. Although it is possible that a monoclonal phase of granulopoiesis was missed in this patient, our results suggest that hypercellular remissions in APL are polyclonal with the elimination of the abnormal clone and regeneration of normal stem cells.

The promyelocytes at relapse, demonstrating the additional abnormal clone to the standard cytogenetic abnormality, were again monoclonal with the same pattern as the promyelocytes at diagnosis. This clone is thus likely to be derived from the original clone and does not represent emergence of a coexistent initial population. Transitional peripheral myeloid cells that appeared after ATRA treatment coincided with the persistence of the abnormal clone.

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**Fig 2.** PGK clonal analysis by Southern blot of APL patient at stages during ATRA treatment. The RFLP identified by digestion with Bst XI and Pst I (a) differentiates the two X chromosomes. Subsequent digestion with methylation-sensitive enzyme Hpa II (b) digests the active form of the X chromosome. Monoclonality, identified by the complete loss of the 0.9-kb allele, was seen in both the leukemic promyelocytes (panel 2) and granulocytes appearing 7 days post-ATRA treatment (panel 3). A normal polyclonal pattern showing equal reduction in both bands is shown by the patients’ lymphocytes (panel 1) and 5 weeks post-ATRA treatment (panel 4).
in 100% of the eight BM metaphases studied. Subsequent clonal analysis of this PMN fraction showed an identical monoclonal pattern to the promyelocytes. This confirms in vivo the differentiative action of ATRA. Interestingly, ATRA was able to differentiate the abnormal promyelocytes despite clonal evolution.

Five weeks after treatment with ATRA the patient entered a complete cytogenetic remission. Serial clonal analysis in parallel showed return to polyclonal granulopoiesis within 5 weeks. This indicates that ATRA likely causes terminal differentiation of APL promyelocytes, leading to clonal elimination and repopulation of the marrow by residual normal stem cells. X-linked probes and methylation analysis allow insight into mechanism of drug action in malignant hematologic disease, and is a particularly powerful methodology to explore differentiation.

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