Terminal Differentiation of Human Promyelocytic Leukemic Cells in Primary Culture in Response to Retinoic Acid

By T. R. Breitman, Steven J. Collins, and B. R. Keene

The recent finding that retinoic acid induces terminal granulocytic differentiation of the human promyelocytic leukemia cell line, HL-60, prompted an investigation of the sensitivity to this inducer of human myelocytic leukemia cells in primary suspension culture. Of the 21 leukemic specimens, only cells from the two patients with acute promyelocytic leukemia differentiated in response to retinoic acid. After an incubation period of 5–7 days in 1 μM retinoic acid, the cells from these two patients showed extensive morphological and functional maturation. Thus, because it appears that retinoic acid specifically induces granulocytic differentiation of leukemic promyelocytes, this compound may have therapeutic utility in the treatment of acute promyelocytic leukemia.

A CUTE myelogenous leukemia has been viewed as a disease involving a block in the normal maturation of myeloid cells.1,2 Certain cultured cell lines, including M-1 murine myeloid leukemia,3,4 Friend murine erythroleukemia,5,6 HL-60 human promyelocytic leukemia,7,8 and K562 human leukemia,9 are induced to mature in vitro by treatment with one or more of a wide variety of agents, including dimethylsulfoxide (DMSO) and other polar-planar compounds, butyric acid, and hypoxanthine. While it has been suggested that such compounds could play a role in the therapy of certain leukemias,10,11 there are several serious drawbacks to this approach. Such compounds are active only at relatively high concentrations and little is known of their pharmacology and toxicity in man. Moreover, it is clear that not all leukemic cell lines respond to these differentiation-inducing agents and among the cell lines that are induced to differentiate, the sensitivity to each compound varies.8,12 Thus, it would seem useful to identify those patients whose leukemic cells can be induced to terminally differentiate before therapy with inducing agents is initiated.

We recently found that retinoic acid is a potent inducer of terminal granulocytic differentiation of the human promyelocytic leukemia cell line, HL-60.12 On a molar basis, this compound is some 106-fold more active than DMSO in inducing differentiation of HL-60. Maximal differentiation (approximately 95%) occurs with 1 μM retinoic acid after 5 days of incubation,12 a concentration pharmacologically obtainable in man.13 Thus, retinoic acid may have use as a differentiation inducing agent in the therapy of human myeloid leukemia.

To determine whether the differentiative response of HL-60 to retinoic acid was shared by other leukemic cells, we incubated fresh human myeloid leukemia cells in short-term suspension cultures in the presence of retinoic acid. Our results indicate that terminal granulocytic differentiation of only promyelocytic leukemia cells is induced by retinoic acid.

MATERIALS AND METHODS

Patient Population

Twenty-one leukemic patients were studied, of whom 3 were in their first relapse and 18 were untreated. The diagnosis was acute myelocytic leukemia in 13 patients, acute promyelocytic leukemia in 2 patients, acute myelomonocytic leukemia in 2 patients, chronic myelocytic leukemia in blastic phase in 3 patients, and chronic myelomonocytic leukemia in accelerated phase in 1 patient. Leukopheresis cells (M.D. Anderson) were shipped on wet ice and prepared in our laboratory the following day. Leukemic bone marrow samples (Baltimore Cancer Research Program) and peripheral blood were received on wet ice and prepared the same day.

Leukocyte Preparation and Incubation

Approximately 3 ml of each sample were diluted to 10 ml with DME/F12 [equal volumes of Dulbecco’s modified Eagle’s medium (MEM) and Ham’s F12 supplemented with 1.2 g of NaHCO3/liter and 15 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes)] and overlaid on 4 ml of Ficoll-Hypaque (Pharmacia, Piscataway, N.J.) and centrifuged at 800 g for 25 min.14 The cells in the interface were removed with a pipette, washed once with phosphate-buffered saline, and unless otherwise indicated, suspended at a concentration of 5 × 10⁴/ml in DME/F12 plus 10% (v/v) heat-inactivated fetal calf serum (Flow Laboratories, McLean, Va.) in 25 sq cm polystyrene flasks (Corning, New York). Cells were incubated for up to 7 days with no change of medium at 37°C in a humidified atmosphere of 5% CO₂ in air. Retinoic acid (all-trans-β-retinoic acid) (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 95% ethanol at a concentration of 1 mM and diluted into the growth media such that the final ethanol concentration was 0.1%. Because this concentration of ethanol has been shown previously15 to have no effect on differentiation of HL-60, an ethanol control was not included in this study. All manipulations involving retinoic acid were performed in subdued light. Cells were counted with a Coulter Counter or in a hemocytometer chamber. Viability was determined by Trypan blue dye exclusion.

Assessment of Maturation

Morphological assessment of the cells was made on Cytospin slide preparations stained with Wright-Giemsa. Differential counts were performed under light microscopy on a minimum of 200 cells.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Age</th>
<th>WBC (x 10^9/liter)</th>
<th>Blast Cells (%)</th>
<th>Sample</th>
<th>Day in Culture</th>
<th>Condition</th>
<th>Bl</th>
<th>Prom</th>
<th>Mye</th>
<th>Granulocyte*</th>
<th>Mono</th>
<th>NBT (%)</th>
<th>Cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>APL</td>
<td>14</td>
<td>0.8</td>
<td>3</td>
<td>BM</td>
<td>0</td>
<td>—</td>
<td>7</td>
<td>89</td>
<td>20</td>
<td>4</td>
<td>0 ND</td>
<td>1.8†</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AML</td>
<td>24</td>
<td>114</td>
<td>96</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>86</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>ND</td>
<td>6.2‡</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CML (BC)</td>
<td>60</td>
<td>151</td>
<td>56</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>86</td>
<td>8</td>
<td>8</td>
<td>0</td>
<td>ND</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CML (BC)</td>
<td>62</td>
<td>185</td>
<td>69</td>
<td>BM</td>
<td>0</td>
<td>—</td>
<td>89</td>
<td>2</td>
<td>8</td>
<td>0</td>
<td>ND</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>AML</td>
<td>72</td>
<td>113</td>
<td>53</td>
<td>BM</td>
<td>0</td>
<td>—</td>
<td>40</td>
<td>56</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>AML</td>
<td>42</td>
<td>3.0</td>
<td>13</td>
<td>BM</td>
<td>0</td>
<td>—</td>
<td>91</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>ND</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>AML</td>
<td>29</td>
<td>1.0</td>
<td>0</td>
<td>BM</td>
<td>0</td>
<td>—</td>
<td>84</td>
<td>5</td>
<td>8</td>
<td>0</td>
<td>ND</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>AML</td>
<td>61</td>
<td>106</td>
<td>96</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>AML</td>
<td>45</td>
<td>25</td>
<td>56</td>
<td>BM</td>
<td>0</td>
<td>—</td>
<td>80</td>
<td>15</td>
<td>2</td>
<td>3</td>
<td>ND</td>
<td>4.6‡</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>AML</td>
<td>67</td>
<td>56</td>
<td>53</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>43</td>
<td>25</td>
<td>18</td>
<td>1</td>
<td>ND</td>
<td>5.0‡</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>AML</td>
<td>48</td>
<td>112</td>
<td>91</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>99</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>5.0‡</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>AMML</td>
<td>77</td>
<td>53</td>
<td>94</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>51</td>
<td>29</td>
<td>11</td>
<td>1</td>
<td>8 15</td>
<td>6.1†</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>AML</td>
<td>69</td>
<td>19</td>
<td>87</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>78</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>ND</td>
<td>2.5‡</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>CMMOL</td>
<td>66</td>
<td>42</td>
<td>21</td>
<td>PB</td>
<td>0</td>
<td>—</td>
<td>33</td>
<td>2</td>
<td>6</td>
<td>55</td>
<td>ND</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>AML</td>
<td>20</td>
<td>38.4</td>
<td>35</td>
<td>BM</td>
<td>0</td>
<td>—</td>
<td>95</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>ND</td>
<td>2.5‡</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>AML</td>
<td>82</td>
<td>25</td>
<td>60</td>
<td>BM</td>
<td>0</td>
<td>—</td>
<td>88</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>ND</td>
<td>3.0†</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>CML (BC)</td>
<td>67</td>
<td>60</td>
<td>50</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>82</td>
<td>3</td>
<td>7</td>
<td>8</td>
<td>ND</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>AML</td>
<td>24</td>
<td>25</td>
<td>34</td>
<td>BM</td>
<td>0</td>
<td>—</td>
<td>18</td>
<td>3</td>
<td>22</td>
<td>6</td>
<td>51</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>AMML</td>
<td>32</td>
<td>129</td>
<td>96</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>91</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>ND</td>
<td>4.5‡</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>AML</td>
<td>55</td>
<td>74.5</td>
<td>5</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>9</td>
<td>5</td>
<td>7</td>
<td>34</td>
<td>45</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>AML</td>
<td>45</td>
<td>240</td>
<td>91</td>
<td>L</td>
<td>0</td>
<td>—</td>
<td>16</td>
<td>37</td>
<td>9</td>
<td>1</td>
<td>37</td>
<td>5.5‡</td>
<td></td>
</tr>
</tbody>
</table>

Ave, acute promyelocytic leukemia; AML, acute myelocytic leukemia; CML, chronic myelocytic leukemia; AMML, acute myelomonocytic leukemia; CMMOL, chronic myelomonocytic leukemia; Bl, myeloblast; Prom, promyelocyte; Mye, myelocyte; Mono, monocyte/macrophage; BM, bone marrow; L, leukemophere; PB, peripheral blood; NBT, nitroblue tetrazolium; RA, retinoic acid; BC, blast crisis; ND, not determined.

*Metamyelocytes, banded and segmented neutrophils.
†Visible cells as determined by trypan blue exclusion.
‡Cells from patients 2, 9, 12, 13, 18, 20, and 21 were seeded on day 0 at a concentration of 5 x 10^3/ml.
Nitroblue tetrazolium (NBT) (Aldrich, Milwaukee, Wisc.) reduction was assayed as described. The percentage of cells containing intracellular blue-black formazan deposits was determined on Wright-Giemsa stained Cytospin slide preparations on at least 200 cells.

RESULTS

The myelogenous leukemia cells derived from 21 patients and incubated for 5–7 days in suspension culture with and without retinoic acid showed little increase and in some cases a decrease in cell concentration (Table I). Viability in both untreated and treated cultures was consistently greater than 75%. This relatively poor proliferative capacity of myelogenous leukemia cells incubated in suspension culture without conditioned media has been noted in other studies.

Differentiation of the leukemic cells from 2 of the 21 patients was induced by retinoic acid (Table I). The only inducible specimens were from the two patients with acute promyelocytic leukemia (patients 1 and 2), both of whose cells incubated in the absence of retinoic acid did not morphologically differentiate and did not reduce NBT (Fig. 1). By day 5 of incubation with 1 μM retinoic acid, 69% of the cells from patient 2 had differentiated into metamyelocytes and banded and segmented neutrophils, and 100% of the cells reduced NBT (Fig. 1). A second sample from this patient from an independent leukopheresis gave identical results. The cells from patient 1 responded more slowly to retinoic acid and were only 48% NBT positive by day 5 but 90% positive by day 7. The cells from both patients showed little growth during incubation with and without retinoic acid although viability remained above 75%. This high percentage of viable cells eliminates the possibility that all or a major part of the retinoic-acid-induced differentiation was a result of selective enrichment for differentiated cells.

None of the other patients' cells showed any significant granulocytic differentiation induced by retinoic acid. In no specimen did retinoic acid significantly affect viability of the total cell counts. Prostaglandin...
E₂ (100 ng/ml) showed no effect on differentiation, either alone or in combination with retinoic acid (data not shown), though this compound has been found to synergistically induce granulocytic differentiation in HL-60 in the presence of retinoic acid (T. R. Breitman and B. R. Keene, unpublished results). In several of the cultures, especially those diagnosed as myelomonocytic leukemia, cells morphologically resembling monocytes/macrophages developed after a few days in suspension culture. These cells were NBT positive, also a functional characteristic of monocytes and macrophages. Similar spontaneous differentiation of fresh leukemic cells in short-term culture has been described. However, retinoic acid had essentially no effect on this spontaneous differentiation.

**DISCUSSION**

Like HL-60, the two specimens induced by retinoic acid were acute promyelocytic leukemias, suggesting that to respond to this compound it is necessary that leukemic cells be at the promyelocytic stage of differentiation. Specific cytologic alterations, as well as a common karyotypic abnormality, also distinguish acute promyelocytic leukemia from other acute leukemias. None of the other 19 less differentiated leukemic specimens showed a response to retinoic acid, just as human myeloid cell lines KG-1 and K-562 also show no response.

It is not known why some cells respond to retinoic acid, while most do not. It has been suggested that the response of different cell types to retinoic acid is mediated through a cytoplasmic retinoic-acid-binding protein. However, we have been unable to demonstrate such a binding protein in HL-60 cells, suggesting that retinoic acid exerts its differentiation effect on leukemic cells by other means.

In both patients 1 and 2, granulocytic differentiation occurred in retinoic-acid-treated cultures without an increase in cell number or a significant loss of cell viability. This would suggest that in these short-term cultures, cell division is not required for differentiation, a finding that has been confirmed with HL-60 (T. R. Breitman and B. R. Keene, unpublished results).

Studies of leukemic cells in soft agar, in diffusion chambers, and in suspension culture suggest at least partial retention of granulocytic or monocytic/macrophage differentiation by at least some of the cells. The therapeutic role of agents that induce differentiation in human leukemia remains in question whether used alone or in combination with conventional chemotherapy, but any factor mediating differentiation of leukemic cells might be expected to favorably affect the progression of the disease. Moreover, all-trans-retinoic acid, which is as active as all-trans-retinoic acid in inducing HL-60, has been used systemically to treat certain skin disorders and is well tolerated. The utility of retinoic acid in this regard remains conjectural and from our results would apparently be limited to patients with acute promyelocytic leukemia, accounting for 13% of acute nonlymphocytic leukemias.

**ACKNOWLEDGMENT**

We thank Dr. R. C. Gallo for his support and Erssel Richardson for expert technical assistance. We thank Dr. Peter Wiernik and Sue Marcus of the Baltimore Cancer Research Program and Drs. Kenneth McCredie and Jean Hester of M. D. Anderson Hospital for the patient samples.

**REFERENCES**


Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid

TR Breitman, SJ Collins and BR Keene