Terminal Differentiation of the Human Promyelocytic Leukemia Cell Line, HL-60, in the Absence of Cell Proliferation

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ABSTRACT

Induction of differentiation of the human promyelocytic leukemia cell line, HL-60, by dimethyl sulfoxide was analyzed for a requirement for cell replication. The ability of HL-60 cells to undergo terminal granulocytic differentiation as judged by nitroblue tetrazolium reduction, phagocytosis, and morphological criteria was not impaired by a total block in cellular proliferation. Retinoic acid, actinomycin D, and butyric acid also induced differentiation of HL-60 cells in the absence of cell growth. These results and the earlier demonstration that phorbol ester-induced macrophage differentiation of HL-60 occurred independently of DNA synthesis indicate that in these leukemic cells there is a dissociation of proliferation and maturation. The ability of retinoic acid to enhance differentiation of HL-60 cells was not altered in the presence of various growth-inhibiting concentrations of two clinically useful chemotherapeutic agents: hydroxyurea and 1-β-D-arabinofuranosylcytosine. These results suggest that combination therapy in a program aimed at both inhibiting proliferation and inducing differentiation of leukemia cells could be beneficial.

INTRODUCTION

It has been suggested that DNA synthesis and a critical number of cell replications are required for the differentiation of specific tissues and cell types. This linkage has been described for erythropoietin-dependent differentiation of both fetal mouse liver (13, 24, 25) and chick embryo erythroblasts (32) and for prolactin stimulation of milk protein synthesis in mouse mammary gland explants (23). However, in other studies, a decrease or absence of cell growth has been associated with cell differentiation, as demonstrated for epidermal keratinocytes (30), for conversion of mouse fibroblasts to adipocytes (12), and for myoblast fusion (4). The relationship between differentiation and cell proliferation has also been studied using Friend erythroleukemia cells (8). Here, too, contradictory results have been reported (10, 15–17, 20).

In an attempt to study this problem further, we have investigated the relationship between differentiation and cell proliferation in the human promyelocytic cell line, HL-60 (6). These leukemic cells can be induced to differentiate into morphologically mature myeloid cells upon addition of various inducing agents (3, 5, 6). In addition, these mature cells display many of the functional characteristics associated with normal peripheral blood granulocytes such as chemotaxis, phagocytosis, and superoxide anion production (7, 22).

In this study, the ability of HL-60 cells to differentiate under a variety of conditions in which cell proliferation is reduced or does not occur was analyzed. The results show that terminal differentiation of HL-60 cells into mature granulocytes does not require cell division.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. HL-60 cells were passaged twice weekly in RPMI-FCS1 in a 7% CO2 humidified atmosphere at 37°C. For the experiments with 2 chemotherapeutic agents, ara-C and hydroxyurea, the cells were grown in a medium consisting of equal volumes of Dulbecco's modified Eagle's minimal essential medium and Ham's F-12 medium supplemented with 1.2 g of NaHCO3 per liter, 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 10% fetal calf serum. Cells were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. All experiments were performed on cells between passages 20 and 70. Cell counts were determined in a Burker chamber or with a Coulter Counter and viability was assessed by trypan blue dye exclusion.

Induction and Assessment of Differentiation. Cells (5 x 10⁶/ml) were exposed to the following inducers at concentrations (unless otherwise indicated) inducing maximal differentiation of exponentially growing HL-60 cells: 180 μM DMSO; 0.6 mM butyric acid; 3.6 mM actinomycin D; and 1 μM RA (3, 5, 6).

The extent of differentiation was assessed by morphology, NBT reduction (29), or phagocytosis (18). Morphological assessment of the cells was made on Cytospin slide preparations stained with Wright-Giemsa. NBT reduction was assayed as described (7), and the percentage of NBT+ cells was determined either on at least 500 cells in a Burker chamber, or on Wright-Giemsa-stained Cytospin slide preparations. Phagocytosis was measured with yeast activated by incubating yeast (2 x 10⁶/ml) in fresh human serum for 20 min at 37°C. The yeasts were then harvested and resuspended in phosphate-buffered saline (138.9 mM NaCl:2.7 mM KCl:8.1 mM Na₂HPO₄:1.5 mM KH₂PO₄) at a concentration of 10⁸/ml. HL-60 cells (10⁶/ml) and yeast (10⁷/ml) were then incubated in Roswell Park Memorial Institute Medium 1640 for 30 min at 37°C. The percentage of cells containing one or more yeasts in their cytoplasm was determined either in a hemocytometer by counting at least 500 cells or on Cytospin slide preparations stained with Wright-Giemsa.

Synchronization of Cells. HL-60 cells were synchronized with respect to the cell cycle by a modification of the method of Puck (26). Briefly, cells were incubated for 24 hr in RPMI-FCS containing 2 mM dThd and then washed and resuspended in dThd-free medium for 10 hr. Then they were again blocked by incubation with 2 mM dThd for 14 hr. At this point (T₀), 3 cultures were seeded as depicted in Chart 1: Culture A was maintained with dThd alone for an additional 48 hr; Culture B was treated with dThd and DMSO for 12 hr and then transferred to medium containing 2 mM dThd for an additional 36 hr (Tₑ); and Culture C was also treated with dThd and DMSO for 12 hr but then was transferred to RPMI-FCS that did not contain either dThd.

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2 To whom requests for reprints should be addressed.

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4421

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or inducer. Because of the possible decrease in dThd activity due to catabolism, the cultures were transferred to fresh medium at T₄₈. In all cultures, the extent of differentiation was determined at T₄₈. The cells were treated with actinomycin D and RA for 24 hr and with butyrinic acid and DMSO for only 12 hr because of the toxicity of these 2 inducers when used in combination with 2 mM dThd.

The extent of synchrony was monitored by determinations of cell number, M₁, and changes in [³H]dThd-labeled DNA per cell. M₁ was determined by counting mitotic figures in 1000 cells in Cytospin slide preparations stained with Wright-Giemsa. The effectiveness of dThd arrest was tested by labeling the DNA of cells with [³H]dThd (2.5 µCi/ml; 6.8 Ci/mmol) during the entire release period after the first dThd block. By this method, all cells going through the S phase were labeled. [³H]dThd-labeled DNA was determined both on the population and on individual cells. For the former, HL-60 cells were washed and then precipitated with 10% trichloroacetic acid. The precipitate was collected on a membrane filter, washed with 5% trichloroacetic acid, and then counted in Insta-Gel (Packard Instrument Co., Downers Grove, III.) in a liquid scintillation counter. dThd incorporation into individual cells was measured by autoradiography as described (1). Cytospin-prepared slides were exposed for 10 days and then counterstained with buffered Giemsa. Mean grain count was determined on 500 cells.

RESULTS

DMSO-induced Differentiation on dThd-blocked HL-60 Cells. The experimental design for induction of differentiation of synchronized HL-60 cells is shown in Chart 1. At the 14th hr of the second dThd block, 3 different cultures were seeded at T₀. Culture A was maintained in medium containing 2 mM dThd from T₀ to T₁₂; Culture B was exposed to DMSO from T₀ to T₁₂ and maintained with 2 mM dThd from T₁₂ to T₄₈; and Culture C was exposed to DMSO and 2 mM dThd from T₀ to T₁₂, and then transferred to DMSO- and dThd-free medium from T₁₂ to T₄₈.

The effectiveness of the inhibition of cell proliferation by 2 mM dThd is demonstrated by the absence, from T₀ to T₄₈, of any increase in the cell number in Cultures A and B or of a dilution of the labeled DNA per cell (measured by 2 different methods) in Culture B (Table 1). In addition, the M₁ in these 2 cultures was <0.3% from T₀ to T₄₈. In contrast, in Culture C, as a result of the removal of dThd at T₁₂, there was at T₄₈ both a doubling in cell number and a 2-fold decrease in labeled DNA per cell (Table 1). Viability was high under all culture conditions. Thus, at T₁₂ viability was >90% for Cultures A, B, and C; at T₄₈, it was >90% for Cultures A and C and >80% for Culture B. These high viabilities eliminate the possibility that all, or a major part of, induced differentiation is a result of selective enrichment for differentiated cells.

Differentiation of HL-60 cells was induced by DMSO under conditions allowing no cell proliferation (Table 1, Culture B). There was an increase in functionally differentiated cells ex-

<table>
<thead>
<tr>
<th>Time in culture (hr)</th>
<th>Viable cells/ml (X 10⁵)</th>
<th>M₁ (%)</th>
<th>cpm/10⁶ cells</th>
<th>Mean grains/cell</th>
<th>NBT⁺ cells (%)</th>
<th>NBT⁺ cells/ml (X 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, B, C T₀</td>
<td>8.1</td>
<td>&lt;0.3</td>
<td>490 ± 20³</td>
<td>18.2</td>
<td>6.1 ± 0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>A T₄₈</td>
<td>7.7</td>
<td>&lt;0.2</td>
<td>ND</td>
<td>ND</td>
<td>19.4 ± 5.4</td>
<td>1.5</td>
</tr>
<tr>
<td>B T₄₈</td>
<td>6.5</td>
<td>&lt;0.2</td>
<td>467 ± 22</td>
<td>18.8</td>
<td>53.6 ± 5.6</td>
<td>3.5</td>
</tr>
<tr>
<td>C T₄₈</td>
<td>16.6</td>
<td>2.2</td>
<td>220 ± 40</td>
<td>10.5</td>
<td>39.1 ± 8.8</td>
<td>6.5</td>
</tr>
</tbody>
</table>

³ Cells prelabeled with [³H]dThd, as described in "Materials and Methods," were assayed for radioactivity at T₀ and T₄₈ by determining both the total cpm in a portion of the culture containing 10⁶ cells and the mean grain number per cell by autoradiography.

⁴ The concentration of NBT⁺ cells was calculated by the formula

\[
\text{NBT⁺ cells/ml} = \frac{\% \text{ of NBT⁺ cells} \times \text{viable cells/ml}}{100}
\]

³ Mean ± S.D. of 3 determinations.
⁴ ND, not done.

Table 1
Induced differentiation of HL-60 by DMSO under conditions of dThd blockage

Chart 1. Experimental design for the induction of differentiation of nonproliferating HL-60. Cells were incubated for 24 hr in RPMI-FCS containing 2 mM dThd and released from the dThd block by transfer to dThd-free medium for 10 hr. They were then blocked again by incubation with 2 mM dThd for 14 hr. At this point (T₁₂), 3 cultures were initiated: Culture A was maintained with 2 mM dThd alone for an additional 48 hr; Culture B was treated with 2 mM dThd for an additional 36 hr (T₄₈); and Culture C was also treated with 2 mM dThd and DMSO for 12 hr but then transferred to medium that did not contain either dThd or DMSO.
pressed either as a percentage of NBT$^+$ cells in the population or as the number of NBT$^+$ cells per ml of culture. These values for Culture B at $T_{48}$ were 53.6% and $3.5 \times 10^5$/ml, respectively, and represent a marked increase compared to the values at $T_0$ when 6.1% of the population and $0.5 \times 10^5$ cells/ml were NBT$^+$. Culture A, treated in a manner identical to that of Culture B, except that there was no exposure to DMSO, also showed an increase in differentiated cells with values for NBT$^+$ cells of 19.4% of the population and $1.5 \times 10^5$/ml. A similar increase in differentiation was reported previously (31) for HL-60 cultures released from a double dThd block. Thus, it appears that a double dThd block of HL-60 results in a small increase in differentiation with or without subsequent cell proliferation.

Treatment of HL-60 with DMSO immediately after the dThd block (31) or during a continuation of a dThd block (Table 1, Culture B) leads to additional differentiation. In addition, treatment of HL-60 with DMSO during a dThd block, followed by a release from both inducer and inhibition of cell proliferation, led to an increase in differentiation with values for NBT$^+$ cells of 39% of the population and $6.5 \times 10^5$ cells/ml (Table 1, Culture C). These high values indicate that a high proportion of the newly formed daughter cells also differentiated.

**Action of Other Differentiation-inducing Agents on Non-proliferating HL-60 Cells.** RA, actinomycin D, and butyric acid also induced differentiation on dThd-blocked HL-60 cells (Table 2). The Culture B condition (Chart 1) for DMSO was used again, except that RA and actinomycin D treatment was for 24 hr instead of 12 hr. There was no increase, and in fact there was a decrease, in the viable cell concentration from $T_0$ to $T_{48}$ (Table 2). In addition, at $T_{48}$, there was no decrease in the radioactivity of cells pre-labeled with $[^{3}H]$dThd before induction and viability ranged from 72% to 87%. Thus, under conditions allowing no cell proliferation, RA, actinomycin D, and butyric acid induced increases in both the percentage of NBT$^+$ cells in the population and the number of NBT$^+$ cells per ml of culture (Table 2).

**Functional and Morphological Differentiation.** In addition to the NBT test, functional differentiation was also assayed by phagocytosis in all cultures at $T_0$ and $T_{48}$. The values for percentages of cells capable of phagocytosis and NBT reduction were similar (data not shown) in agreement with results reported previously for DMSO-induced HL-60 cells (31). Recognizable morphological changes also occurred after induction. The concentration (cells $\times 10^5$/ml) of mature cells (defined as myelocytes, metamyelocytes, and neutrophilic granulocytes) increased from 0.4 to 0.5 at $T_0$ to 1.4 with actinomycin D or butyric acid, to 2.1 with DMSO, and to 3.4 with RA for cultures incubated under Condition B, outlined in Tables 1 and 2. These values are lower than those observed with either NBT reduction (Tables 1 and 2) or phagocytosis. These differences have been observed by others (18, 31) and probably reflect the subjective nature of assigning a morphological designation to cells that are intermediates between the promyelocyte and myelocyte stages of differentiation, since others have reported almost identical values based on either morphology or function (5, 7).

**RA-induced Differentiation of HL-60 Cells Growth Inhibited by Hydroxyurea and ara-C.** RA was tested in combination with 2 chemotherapeutic agents, hydroxyurea and ara-C. Both of these agents are known to inhibit cell growth by specifically inhibiting DNA synthesis (21, 33). HL-60 cells were grown at various concentrations of the drug, with and without RA. The concentration of hydroxyurea that inhibited growth by 50% was 100 $\mu$M for cultures growing either with or without 1 $\mu$M RA (Chart 2). RA alone, as described previously (3), decreased growth, and increasing concentrations of hydroxyurea decreased the cell number still further. However, hydroxyurea inhibition of cell growth had essentially no effect on the extent of RA-induced differentiation of HL-60 (Chart 3), as measured by the concentration of mature (NBT$^+$) cells, as a function of the total cell concentration (Chart 3). Thus, in the presence of 1 $\mu$M RA, there was essentially a constant value of 0.66 for the

---

**Table 2**

<table>
<thead>
<tr>
<th>Inducer</th>
<th>$T_0$</th>
<th>$T_{48}$</th>
<th>$T_0$</th>
<th>$T_{48}$</th>
<th>$T_0$</th>
<th>$T_{48}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric acid</td>
<td>7.2</td>
<td>6.3</td>
<td>5.6</td>
<td>46.5 $\pm$ 4.9$^b$</td>
<td>0.4</td>
<td>2.9</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>7.2</td>
<td>5.5</td>
<td>5.6</td>
<td>49 $\pm$ 4.3</td>
<td>0.4</td>
<td>2.7</td>
</tr>
<tr>
<td>RA</td>
<td>7.2</td>
<td>5.2</td>
<td>5.6</td>
<td>86 $\pm$ 7.6</td>
<td>0.4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

$^a$ The concentration of NBT$^+$ cells was calculated by the formula

$$\text{NBT}^+ \text{cells/ml} = \frac{\% \text{ of NBT}^+ \text{cells} \times \text{viable cells/ml}}{100}$$

$^b$ Mean $\pm$ S.D. of 3 determinations.

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**Chart 2.** Inhibition of growth of HL-60 cells by various concentrations of hydroxyurea in the presence (Ø) or absence (O) of 1 $\mu$M RA.
D. Ferrerò et al.

Chart 3. RA-induced differentiation of HL-60 cells in the presence of various concentrations of hydroxyurea. •, △, ▽, with 1 μM RA; ○, □, △, ▽, no RA. Hydroxyurea concentrations were: none (○, □); 50 μM (●, △); 100 μM (Δ, ▽); 200 μM (▽, ▽). Mature cells were calculated by the formula:

\[
\text{Mature cells/ml} = \frac{\% \text{ of NBT}^+ \text{ cells} \times \text{viable cells/ml}}{100}
\]

Chart 4. Inhibition of growth of HL-60 cells by various concentrations of ara-C in the presence (●) or absence (○) of 0.3 μM RA.

Chart 5. RA-induced differentiation of HL-60 cells in the presence of various concentrations of ara-C. •, △, ▽, with 0.3 μM RA; ○, □, △, ▽, no RA. ara-C concentrations were: none (○, □); 0.1 μM (●, △); 0.3 μM (Δ, ▽); 1 μM (▽, ▽). Mature cells were calculated by the formula:

\[
\text{Mature cells/ml} = \frac{\% \text{ of NBT}^+ \text{ cells} \times \text{viable cells/ml}}{100}
\]

mature cells:total cell ratio, with increasing concentration of hydroxyurea and decreased proliferation (Chart 3). Growth inhibition by hydroxyurea alone had little if any effect on the extent of HL-60 differentiation.

In a similar study with various concentrations of ara-C in both the presence and the absence of 0.3 μM RA, the extent of RA-induced differentiation of HL-60 was not markedly reduced by growth-inhibiting concentrations of ara-C (Charts 4 and 5). In addition, ara-C alone appeared to have an inductive effect on HL-60. Thus, at the highest concentration of ara-C, the culture contained approximately 50% NBT+ cells (0.4 x 10^5 NBT+ cells/ml/0.8 x 10^5 total cells/ml). However, at the initiation of the experiment, there were 0.2 x 10^5 NBT+ cells/ml (8% NBT+ cells x 2.5 x 10^5 cells/ml). These nonproliferating mature cells would be resistant to ara-C with the result that ara-C would selectively kill proliferating cells with a consequent enrichment of the population for NBT+ cells. Thus, most if not all of the increase in the percentage of NBT+ cells in cultures grown with ara-C alone is probably the result of enrichment and not induction. This conclusion is in agreement with the results of others (5, 19) who found little or no morphological maturation of HL-60 at ara-C concentrations lower than 1 μM.

DISCUSSION

The HL-60 cell line is a useful system for biological and molecular analysis of myeloid differentiation (9). The leukemic promyelocytes of this cell line differentiate to mature myeloid cells in the presence of different inducing agents.

In this study, we investigated whether differentiation and cell proliferation of HL-60 cells are inseparable. This was initiated to gain further insight into the mechanism of induction of leukemic cell differentiation. In addition, because RA may have clinical utility (2), it was of interest to examine whether RA exerts its effect on differentiation when added in combination with chemotherapeutic agents that inhibit cell growth.

In a previous study (31), we demonstrated that commitment
to differentiation of HL-60 cells and expression of differentiated functions are 2 distinct phenomena, as observed previously in the Friend system (14). In addition, commitment to differentiation had no linkage to the cell cycle. Here, we found that the entire process of HL-60 differentiation does not require cell division.

The ability of HL-60 to differentiate in the presence of DMSO under conditions of inhibited cell proliferation was analyzed. Blockage of proliferation was achieved by exposing the cells to a high concentration of dThd. Its effectiveness on the blockage was shown by the lack of cell growth (Table 1) and the absence of mitotic figures. In addition, with cells prelabeled with [3H]dThd, there was no decrease in the mean radioactivity per cell during differentiation. A decrease would have been expected if cellular divisions had occurred. In this study, dThd-blocked HL-60 cells were over 50% differentiated after treatment with DMSO for only 12 hr (Table 1, Culture B). Similar results were obtained with other inducers such as RA, actinomycin D, and butyric acid (Table 2). These compounds are chemically different, and a distinct mechanism of action has been suggested for some of them in another system of differentiation, the Friend erythroleukemia cell line (11, 28). In our experiments, cells were blocked by dThd at the beginning of the S phase (31). The possibility that this point of the cell cycle could play an important role in the commitment of the cells to undergo terminal differentiation is unlikely because of the previous demonstration that there is no linkage between commitment to differentiation and phases of the cell cycle (31).

Recently, it was demonstrated that 12-O-tetradecanoylphorbol-13-acetate-directed, macrophage-like differentiation occurs in the absence of DNA synthesis (27). On the basis of these results, we can draw the conclusion that differentiation towards either lineage in the absence of cell proliferation is a biological characteristic of HL-60 cells rather than an effect of a particular inducing agent. This finding suggests dissociation in HL-60 between cell proliferation and maturation. It remains unclear from our experiments whether this dissociation can be ascribed to the partial commitment towards the myeloid differentiation of the HL-60 promyelocytes. Similar studies using less differentiated myeloid cell lines might give further understanding of the relationship between cell proliferation and cell differentiation.

Since there is a dissociation between proliferation and differentiation in HL-60, we tested the possibility of inducing differentiation on HL-60 cells blocked in their proliferative activity by 2 clinically used anticancer agents, hydroxyurea and ara-C. These, as well as other chemotherapeutic agents, have been analyzed for their inducing effect on HL-60 cell differentiation (5, 19). However, the well-known effect of these compounds is to inhibit cell growth by specifically inhibiting DNA synthesis. The concentrations at which these drugs were tested are analogous to their plasma levels when they are used as antileukemic agents. RA was used as an inducing agent because of its possible clinical utility, since it has been observed that it induced differentiation in 2 of 2 cases of primary cultures of fresh leukemic promyelocytes (2). Our results indicate that neither hydroxyurea nor ara-C interferes with the differentiative action of RA and that RA does not interfere with their growth-inhibitory activity. Thus, RA and other retinoids can be considered for combination therapy in a program aimed at both inhibiting proliferation and inducing terminal differentiation of promyelocytic and possibly other immature leukemia cells.

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