Phorbol Ester-induced Differentiation of Human T-Lymphoblastic Cell Line HPB-ALL

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ABSTRACT

12-O-Tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter, induced phenotypic differentiation in the human thymic acute lymphocytic leukemia cell line, HPB-ALL. Within 30 min of seeding in the presence of TPA, the cells formed a smooth round shape. After a 7-day exposure to TPA, the majority of the cells became smaller and reminiscent of large or atypical lymphocytes. Electron microscopic analysis evidenced morphological differentiation in TPA-treated HPB-ALL cells. Thymic antigens stained with monoclonal antibody OKT6 were dramatically reduced while Leu2a-positive cells were increased in the TPA-treated HPB-ALL cells. However, OKT3-positive cells did not appear in these TPA-treated cells for up to 7 days. Upon TPA-induced phenotypic differentiation, the growth rate of cells was significantly inhibited, their ability to incorporate DNA and RNA via 3H-labeled precursors was reduced, their capacity to bind sheep red cell rosettes was significantly increased, and the proportion of terminal deoxynucleotidyl transferase-positive cells was decreased.

INTRODUCTION

TPA has pleomorphic effects on the differentiation and proliferation of normal and malignant cells in vitro (3) and induces terminal differentiation in murine (7) and human myeloid leukemia cells (4, 5, 17). In contrast, little is known of the induction of differentiation in malignant lymphoblasts. However, recent progress in the characterization of markers of T-cell subsets and thymocytes using monoclonal antibodies and histochemical analysis makes feasible investigations of the differentiation induction in human lymphoid cancers (1). We used TPA to test the ability of human T-cell leukemic cell lines to differentiate. Malignant blasts from the various forms of acute leukemia are presumably "frozen" in the early stages of phenotypic reversion in normal cell differentiation. Therefore, differentiation induction in these cells may be of therapeutic value and may also provide insights into the mechanism of differentiation and the origin and pathogenesis of leukemias.

MATERIALS AND METHODS

Cells and Cell Culture. The T-lymphoblast cell line, HPB-ALL, was originally established from the peripheral blood of a 14-year-old Japanese boy with thymic ALL (9, 12). HPB-ALL cells have common T-lymphoblast characteristics such as SRBC rosette formation, positive TdT activity, and common thymocyte antigens (9, 12). The cells were maintained in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal calf serum (Flow Laboratories, Rockville, Md.), penicillin (100 units/ml), and streptomycin (100 mg/ml). In preliminary experiments, the cells were seeded in culture flasks (25 cm2) (Corning Glass Works, Corning, N. Y.) and cultured in the presence of different concentrations of TPA.

Peripheral lymphocytes were isolated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) from heparinized blood samples of healthy donors (11) and used for controls in SRBC rosette formation.

Chemicals. TPA, obtained from Sigma Chemical Co. (St. Louis, Mo.), was dissolved in DMSO (Sigma); this stock solution was stored at −20° and diluted with the culture medium just before use. The final concentration of DMSO in the culture medium was less than 0.005%. The amount of DMSO added to the cultures had no detectable effect on the HPB-ALL cells.

Cell Growth Measurements. Duplicate cultures were carried for each of the 7 culture days. Three different concentrations of TPA were added to the culture medium. Cell number and viability were determined by a hemocytometer in the presence of 2% trypan blue.

Morphological Studies. TPA-treated HPB-ALL cells were examined for morphological changes under a light microscope and a phase-contrast microscope (Nikon, Tokyo, Japan). Electron microscopic study was done as described previously (19).

SRBC Rosette Assay. SRBC rosette formation was determined as described previously (9, 12). As a positive control for SRBC rosette formation, normal human peripheral lymphocytes were used.

TdT Activity. TdT-containing cells were identified by a single-cell indirect immunofluorescence assay using specific rabbit anti-TdT serum (Bethesda Research Laboratories, Gaithersburg, Md.).

Cell Surface Antigens. To assess the qualitative and quantitative changes in TPA-treated HPB-ALL cells, monoclonal anti-T-cell antibodies, OKT3, OKT4, OKT8 (Ortho Diagnostic Systems, Raritan, N. J.); OKT9 and OKT10 (kindly provided by Dr. G. Goldatein, Ortho Diagnostic Systems); and Leu2a and Leu3a (Becton Dickinson, Sunnyvale, Calif.) were used in fluorescence-activated cell sorter system (Model IV; Becton Dickinson). Monoclonal antibodies were titrated to determine the saturated fluorescence intensity level for each antigen concerned and then evaluated optimal concentrations. We used them at 1:125 to 1:250 dilutions as final concentrations. TPA-treated and untreated HPB-ALL cells were stained by the indirect membrane immunofluorescence technique, using monoclonal antibodies and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, Pa.). Twenty thousand cells were counted.
DNA and RNA Synthesis. TPA-treated and untreated cells were continuously labeled with [3H]thymidine (0.5 μCi/ml; New England Nuclear, Boston, Mass.) or [3H]uridine (0.5 μCi/ml; 25 Ci/mmol; New England Nuclear), harvested by centrifugation, precipitated with 5% trichloroacetic acid, and washed with methanol as indicated. The radioactivity of NCS (Amersham-Searle, Evanston, Ill.)-solubilized pellets was measured in a liquid scintillation counter (Packard Instruments) as described previously (9).

RESULTS

Morphological Changes. Figs. 1 to 3 show the morphological changes of HPB-ALL cells during a 7-day TPA exposure. Within 30 min of seeding in the presence of 10 nM TPA, most HPB-ALL cells were smooth and round (Fig. 1). After 7 days of exposure, May-Grünwald-Giemsa-stained cytocentrifuge preparations revealed the disappearance of the formerly prominent nucleolus, a change in the chromatin network, and an increase in cytoplasmic granules. Most of the cells diminished in size and were reminiscent of large or atypical lymphocytes (data not shown). Phase-contrast microscopy revealed the TPA-induced morphological changes to consist of a decrease in cell size, nucleolar disappearance, and a change in the chromatin network (Fig. 2). These changes were confirmed electron microscopically (Fig. 3). The cells lost the characteristic lymphoblast structure and exhibited differential stages from large to small lymphocytes. The trypan blue exclusion test showed that more than 80% of the TPA-treated and untreated HPB-ALL cells were viable 7 days after the start of culture. The maximum effect was noted at a final concentration of 10 nM TPA in the medium. Therefore, unless otherwise noted, this concentration was used throughout.

Effect of TPA on Cell Proliferation. As shown in Chart 1, each of the TPA concentrations used inhibited the growth rate of HPB-ALL cells; at 10 nM TPA, cell proliferation was inhibited more effectively than at 100 nM TPA. Cell viability exceeded 90% in each flask.

Effect of TPA on SRBC Rosette-forming Ability. The ratio of SRBC rosette-forming cells was significantly increased in TPA-treated HPB-ALL cells; this effect became apparent on Day 3 (Table 1).

Effect of TPA on the Ratio of TdT-positive Cells. The ratio of TdT-positive cells was gradually reduced after 3 days of TPA exposure and was significantly lower on Day 7. In untreated control cultures, 30 to 37% of the cells were TdT positive (Table 1).

Effect of TPA on Cell Surface Antigenic Phenotype Changes. The surface of TPA-untreated HPB-ALL cells was positive for OKT4, OKT6, OKT9, OKT10, and Leu3a. Cell surface antigenic phenotype changes induced by TPA are shown in Chart 2 and Fig. 4. OKT6-positive cells predominated among untreated HPB-ALL cells; this antigen was markedly reduced upon TPA treatment (Fig. 4). Since OKT6 is a common thymocyte antigen (15), this finding warranted further investigation. As shown in Table 2, exposure to TPA brought about a remarkable reduction in OKT4-, OKT9- and Leu3a-positive cells. The fluorescence intensity of OKT10-positive cells was increased (Chart 2), but the proportion of fluorescence-positive cells was not changed (Table 2). Therefore, we examined the appearance of other T-cell differentiation antigens using monoclonal T-cell antibodies, i.e., OKT3, which react with peripheral T-cells and OKT8 and Leu2a which are dominant in suppressor-cytotoxic T-cells (14). Whereas the proportion of Leu2a-positive cells was markedly increased after a 7-day TPA exposure, the other antigens did not appear within the observation period (Chart 2). Examination of the time course changes of 3 T-cell antigens, determined by OKT6, Leu2a, and Leu3a, in TPA-treated HPB-ALL cells (Table 1) revealed that antigenic phenotype changes were already apparent on Day 1. The proportion of OKT6-positive cells decreased gradually over time while the number of Leu2a- and Leu3a-positive cells changed rapidly on Day 1 and Day 3, respectively.

Effect of TPA on RNA and DNA Synthesis. As shown in Charts 3 and 4, the ability of HPB-ALL cells to incorporate RNA and DNA via their 3H-labeled precursors was reduced in the presence of TPA. This inhibition of RNA and DNA synthesis

![Chart 1. Growth curve of HPB-ALL cells in Roswell Park Memorial Institute Medium 1640 containing various concentrations of TPA. Each point indicates the mean value of duplicate assays. O, 1 nM TPA; □, 10 nM TPA; △, 100 nM TPA; ●, no TPA.](image-url)

Table 1

<table>
<thead>
<tr>
<th>Time after incubation (days)</th>
<th>SRBC rosette-positive cells (%)</th>
<th>TdT-positive cells (%)</th>
<th>OKT6-positive cells (%)</th>
<th>Leu2a-positive cells (%)</th>
<th>Leu3a-positive cells (%)</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>Control: 24.7 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TPA-treated: 23.1 ± 3.4</td>
<td>Control: 37.2 ± 2.6</td>
<td>TPA-treated: 36.5 ± 3.4</td>
<td>Control: 98.8 ± 3.8</td>
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<tr>
<td>1</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>Control: 30.0 ± 3.8</td>
<td>TPA-treated: 27.7 ± 5.4</td>
<td>Control: 90.8 ± 5.3</td>
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<tr>
<td>3</td>
<td>19.6 ± 3.1</td>
<td>46.9 ± 3.4</td>
<td>35.8 ± 4.1</td>
<td>22.6 ± 1.6</td>
<td>96.8 ± 3.6</td>
</tr>
<tr>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td>32.3 ± 3.4</td>
<td>9.5 ± 1.4</td>
<td>96.3 ± 4.0</td>
</tr>
<tr>
<td>7</td>
<td>23.8 ± 4.5</td>
<td>50.2 ± 8.2</td>
<td>31.4 ± 3.1</td>
<td>6.8 ± 3.3</td>
<td>95.8 ± 4.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> In our assay system, 70.0 ± 8.0 SRBC rosette-positive cells were detected in peripheral blood lymphocytes obtained from healthy adults.

<sup>b</sup> Mean ± S.E. in 3 different experiments.

<sup>c</sup> ND, not done.
Chart 2. Fluorescence-activated antibody cell sorter analysis of cell surface antigenic phenotype changes. Two-dimensional immunofluorescence profiles of untreated and TPA-treated HPB-ALL cells are presented. , control (without monoclonal antibody), , TPA-treated; , untreated. The percentage of fluorescence-positive cells was calculated using a computer system of a Model IV fluorescence-activated cell sorter; for results, see Table 2.

Table 2

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Untreated HPB-ALL Cells (%)</th>
<th>TPA-treated HPB-ALL Cells (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>OKT3</td>
<td>1.7</td>
<td>0.0</td>
</tr>
<tr>
<td>OKT4</td>
<td>83.3</td>
<td>0.0</td>
</tr>
<tr>
<td>OKT6</td>
<td>93.8</td>
<td>30.1</td>
</tr>
<tr>
<td>OKT8</td>
<td>0.7</td>
<td>0.0</td>
</tr>
<tr>
<td>OKT9</td>
<td>96.0</td>
<td>3.5</td>
</tr>
<tr>
<td>OKT10</td>
<td>88.0</td>
<td>88.5</td>
</tr>
<tr>
<td>Leu2a</td>
<td>3.9</td>
<td>25.9</td>
</tr>
<tr>
<td>Leu3a</td>
<td>93.3</td>
<td>4.0</td>
</tr>
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</table>

Paralleled the growth inhibition of HPB-ALL cells; however, they were not accompanied by a significant drop in cell viability.

DISCUSSION

In contrast to myeloid leukemic cells such as HL-60 (2, 16, 18), little is known about the induction of differentiation in malignant lymphoblasts. Nagasawa and Mak (10) reported that TPA induced differentiation in the human T-lymphoblastic cell line Molt-3. Monoclonal antibodies such as the OKT and Leu series have been used to examine cell surface antigenic markers of T-cell lineage during normal differentiation (14, 20). Examination of transmembrane controls in the growth and differentiation of eukaryotic cells should be a high priority. Therefore, a model system of T-lymphoblast differentiation is needed in which the malignant phenotypic reversion can be studied. In the present investigation, we examined the differentiation ability of the thymic ALL cell line HPB-ALL.

We demonstrated that, as defined by morphological changes, loss of TdT activity, increased SRBC rosetting activity, and the disappearance of thymocyte antigen OKT6 (15), HPB-ALL cells differentiated into more mature T-cells upon TPA treatment. The antigenic change may be interpreted as a shedding of membrane antigen or a modulation of antigenicity (13). In conjunction with the observed morphological changes, however, it suggests the induction of T-lymphoblast differentiation by TPA. Furthermore, the proportion of Leu2a-positive cells was markedly increased. This and concomitant reduction in the enzyme activity of nuclear poly(ADP-ribose)⁴ support the hypothesis that T-lymphoblast differentiation takes place in HPB-ALL cells. The later evidence coincides with observations made in the murine myeloid leukemia cell line M1 (6). On the basis of these considerations, we expected the occurrence of terminal differentiation in TPA-treated HPB-ALL cells and examined the appearance of other differentiation antigens de-

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Chart 3. Effect of TPA treatment on RNA synthesis of HPB-ALL cells. TPA-treated and untreated cells were continuously labeled with [3H]thymidine. The values are the mean cpm/tube in duplicate tests. Bars: S.E. @, untreated cells; @, 10 nM TPA-treated cells; @, 100 nM TPA-treated cells; @, 1000 nM TPA-treated cells.

Chart 4. Effect of TPA treatment on DNA synthesis of HPB-ALL cells. TPA-treated and untreated cells were continuously labeled with [3H]thymidine. The values are the mean cpm/tube in duplicate tests. Bars: S.E. @, untreated cells; @, 10 nM TPA-treated cells; @, 100 nM TPA-treated cells; @, 1000 nM TPA-treated cells.

ACKNOWLEDGMENTS

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REFERENCES

18. Sachs, L. Control of normal cell differentiation and the phenotypic reversion al. (21) on chronic lymphocytic leukemia cells. TPA is a potent mitogen for human peripheral blood T-cells (3), and its apparent 2-directional effect suggests that it may act on sites or metabolic processes that approximate a switching mechanism which regulates the onset of cell replication. Therefore, for the occurrence of terminal differentiation in TPA-treated HPB-ALL cells, an additional signal may be required to activate the genes controlling normal phenotypic differentiation. To test the validity of this hypothesis on the multifactorial potency of TPA-induced HPB-ALL differentiation, prolonged cell culture probably should be done. We are testing other substances such as thymic hormones and T-lymphokines for this purpose.

Fig. 1. HPB-ALL cells during the acute phase of morphological changes demonstrated on phase-contrast micrographs of viable untreated (A) and TPA-treated (B) cells. The cells became round within 30 min of exposure to 10 nM TPA. Photographed at 3 hr. x 100.
Fig. 2. Phase-contrast micrographs demonstrating the morphology of untreated (A) and TPA-treated (B) HPB-ALL cells. After a 7-day exposure to 10 nM TPA, the cells lost characteristic lymphoblast structure and resembled large or small lymphocytes with small nuclei encircled by thick nuclear membrane. Some cells exhibited pseudopodia-like protrusions and resembled an atypical lymphocyte. x 400.
Fig. 3. Electron micrographs of untreated (A) and TPA-treated (B) HPB-ALL cells. × 10,000. A. TPA-untreated HPB-ALL cell. The pleomorphic cell body manifests a large eccentrically located nucleus with prominent nucleoli and scanty chromatin aggregates. The cytoplasm is rich in polysomes and contains a small amount of organelles. B. HPB-ALL cell after a 7-day exposure to 10 nm TPA. The cell becomes round but has many cytoplasmic projections. The cytoplasm contains increased numbers of mitochondria, rough endoplasmic reticulum, lipid droplets, and glycogen granules. Aggregates of heterochromatin increased in lobulated nucleus and nucleoli become inconspicuous.
Fig. 4. Fluorescence-activated cell sorter (FACS) analysis of HPB-ALL cell surface antigenic phenotype changes. Untreated (A) and TPA-treated (B) HPB-ALL cells were stained by the indirect membrane immunofluorescence technique, using OKT6 and fluorescein isothiocyanate-conjugated goat anti-mouse IgG. A 3-dimensional histogram of OKT6-positive cells is presented. x-axis (horizontal), distribution of cell size; y-axis (vertical), intensity of fluorescence; z-axis (upward), number of cells. A, OKT6-positive cells prominent in the TPA-untreated sample; B, significant reduction of the antigen on the surface of TPA-treated cells after a 7-day exposure to 10 nm TPA.
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