Induction by Tumor-promoting Phorbol Diester of Colony-stimulating Activity in Human Myeloid Leukemia Cells Transformed to Macrophage-mimicking Cells

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ABSTRACT

The tumor-promoting phorbol diester, 12-O-tetradecanoylphorbol-13-acetate (TPA), appears to induce a macrophage-mimicking transformation of cells in human promyelocytic leukemia constant cultured line HL-60, cells from normal human and chronic myeloid leukemia bone marrow, and cells from human acute leukemias. In the present experiments, peripheral blood and/or bone marrow nucleated cells from 12 patients with acute myeloblastic leukemia (AML), 1 with acute promyelocytic leukemia (APL), 1 with acute myelomonocytic leukemia, 3 with myeloid blast crisis of chronic myelogenous leukemia (BC-CML), and 2 with acute lymphocytic leukemia (ALL) were exposed to TPA at concentrations from 10^{-7} to 10^{-9} M. After 1 to 4 days, in all cases except ALL, the cells acquired the peculiar morphology of macrophage-mimicking cells with multiple long or short thin pseudopods.

We studied the ability of leukemia promyelocytes from HL-60 cells and cells from AML, APL, and BC-CML to produce colony-stimulating activity (CSA) after treatment with TPA. Several samples of HL-60 cells and blasts from leukemia patients were treated with 10^{-8} M TPA in liquid culture for 2 to 6 days and transferred to semisolid agar in which they were maintained for 7 to 24 days. Afterwards, cells in semisolid agar were used as a feeder layer in double-layer semisolid agar colony-forming unit colony formation assay. The formation of normal granulocyte-macrophage colony-forming unit colonies was induced by feeder layers from leukemic cells treated with TPA. Depletion of adherent cells from leukemic cell buffy coats before treatment with TPA did not affect the production of CSA. The intensity of colony formation induced by feeder layers from TPA-treated leukemic cells was comparable to that induced by normal buffy coat feeder layers.

TPA-untreated leukemic cells (HL-60, AML, APL, and BC-CML) did not produce CSA in the same experiments. ALL cells, whether treated with 10^{-8} M TPA or left untreated, did not produce CSA. No CSA was shown by lymphocyte-enriched normal human buffy coats treated with 10^{-8} M TPA. The data showed that CSA production occurs in leukemic blasts treated with TPA themselves but not by some cellular minority present in leukemic blood and bone marrow buffy coats and possibly activated by TPA. TPA (10^{-9} M) itself incubated in culture medium without any cells up to 7 days did not possess any CSA.

These experiments show that TPA is able not only to transform leukemic myeloid cells to macrophage-mimicking cells but also to induce CSA production by these cells.

INTRODUCTION

Chemical induction of differentiation of leukemic cells discovered by Friend et al. (7, 8) and Sachs et al. (32, 40) is a perspective approach for experimental therapy. Now, chemical induction of differentiation is well established for several systems (for review, see Refs. 7 and 40). Tumor-promoting phorbol diesters, in particular TPA, are active modulators of cell differentiation (47). TPA appears to induce a macrophage-mimicking transformation of cells in human promyelocytic leukemia constant culture line HL-60 (37, 39), cells from normal human and CML bone marrow (44), and cells from AML and APL patients (17, 33, 45).

In the presence of TPA, normal cells and myeloid leukemic cells acquire a peculiar shape and stick to the bottom of plastic flasks (MMCs). The present paper reports experiments concerning whether MMCs behave as macrophages in cellular interactions, i.e., whether they acquire such fundamental properties of the monocyte-macrophage lineage as elaboration of CSA. It was previously well established (10, 11) that AML WBCs do not possess any CSA.

MATERIALS AND METHODS

Patients. Eighteen leukemic patients were studied before any treatment or during relapse before new chemotherapy had begun. Twelve patients had AML, 1 APL, 1 AMML, 3 BI-CML, and 1 ALL. Leukemias were diagnosed on the basis of cellular morphology, cytochemical staining including determination of terminal deoxynucleotidyl transferase, and study of immunological markers. All patients except one had more than 80% blasts in the peripheral blood. One patient (V.) with BC-CML had 60% blasts. Cells of all patients were studied for macrophage-mimicking transformation under treatment with TPA, and cells of 7 of them were studied for induction of CSA.

Human Leukemic Cell Line. HL-60 (9) was the gift of Dr. S. J. Collins to Dr. N. Mendelsohn. HL-60 cells were maintained in suspen--
tion culture in RPMI 1640, supplemented with 20% heat-inactivated fetal bovine serum. The culture was passaged every 7 to 10 days.

**Schedule of Experiments.** An outline of the experiments performed is depicted in Chart 1. Peripheral WBCs or bone marrow buffy coat cells obtained from the patients described and cultured cells of the HL-60 promyelocytic leukemia cell line were incubated with TPA (usually $10^{-8}$ M) in liquid cultures for 2 to 14 days. The picture of morphological transformation to MMCs was observed daily. Most TPA-treated myeloid cells acquired typical MMC shape. Then, TPA-treated and TPA-untreated cells (controls) were placed in semisolid agar and cultivated for 7 to 24 days at $37^\circ$ humidified atmosphere with 5% CO$_2$. Using these cells as a feeder layer, the conventional CFU(c) assay was performed. Cells of normal human or CML bone marrow ($10^6$) were placed as an indicator upper layer, and CFU(c) formation was determined.

As controls for the TPA cells in the feeder layers, we have used: (a) untreated blood and bone marrow cells incubated for the same period and under the same experimental conditions as TPA-treated cells; (b) blood and bone marrow cells treated by small doses of TPA, mostly $10^{-11}$ M; (c) lymphocyte-enriched buffy coat (85% of lymphocytes) obtained through leukopheresis of normal donors treated by TPA in doses of $10^{-8}$ M and $10^{-11}$ M; (d) $10^{-8}$ M TPA incubated in flasks with culture medium without cells; (e) $10^{-8}$ M TPA added directly to the bottom agar layer; and (f) human ALL cells cultivated with $10^{-8}$ M TPA.

The CSA produced by TPA-treated myeloid leukemic blasts was compared to that produced by normal human blood buffy coat feeder layer ($10^6$ WBCs) and to a standard urinary bladder carcinoma cell line 5637-conditioned media feeder layer (46).

**SCHEDULE OF EXPERIMENTS**

<table>
<thead>
<tr>
<th>Experiment Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffy coat</td>
<td>Upper layer normal or CML marrow</td>
</tr>
<tr>
<td>Depletion of adherent cells</td>
<td>2 hours</td>
</tr>
<tr>
<td>Cells used as feeder layer in semi-solid agar</td>
<td></td>
</tr>
<tr>
<td>Liquid cultures with $10^{-8}$ M TPA</td>
<td>2 days</td>
</tr>
</tbody>
</table>

**TPA Preparation.** TPA was purchased from Peter Borchert, chemical carcinogenesis, University of Minnesota. From a stock solution of TPA in acetone ($2 \times 10^{-4}$ M) stored at $-70^\circ$, we made serial dilutions in the culture medium.

**Cells.** Bone marrow cells were obtained from iliac punctures using syringes containing preservative-free heparin. Cells were centrifuged at 750 x g for 15 min. Buffy coat was collected and washed twice either with RPMI 1640 or McCoy's 5A culture medium. Viable cells were plated into 75-sq cm culture flasks (Falcon) in a volume of 20 ml in a concentration of $2 \times 10^6$ nucleated cells/ml of RPMI 1640 with 20% heat-inactivated fetal bovine serum containing 100 units of penicillin and 100 $\mu$g of streptomycin per ml. Blood samples were taken by venipuncture with syringes containing the same heparin solution. The tubes with blood were usually left at room temperature for about 1 hr. During this time, the RBCs settled, and the WBCs formed a thick upper layer. The upper layer was then centrifuged for 10 min at 750 x g. Then, it was washed twice in culture medium. The washed cells were placed in 75-sq cm flasks in the same concentration and conditions as bone marrow cells.

**Cell Cultures.** The bone marrow and blood cells were kept in plastic Petri dishes for 2 hr before liquid culture use. This was done to eliminate the adherent cells from the suspension to avoid their potential for CSA production. One half of the remaining nonadherent cells were then treated with TPA; the other half remained as controls. The cultures with TPA and the controls were incubated at $37^\circ$ in a humidified atmosphere with 5% CO$_2$ for 2 to 3 days (see tables). After incubation, culture fluids with floating cells were harvested, and the flasks were additionally washed. The cells which were attached to the bottom were dislodged by banging or agitation with the addition of a small amount of medium.

The supernatant and suspended detached cells were combined and centrifuged at 750 x g for 10 min. The cells were resuspended, counted, and used for further manipulations.

**CFU(c) assay in semisolid agar** was performed according to a modification (28) of previously described methods (2, 27, 34, 35) and was used for determining the production of CSA by the feeder layers of leukemic cells treated with TPA. For these purposes, $10^6$ treated cells were placed in 0.5% agar/35-mm plate. Others have usually used their normal buffy coat feeder layers for CFU(c) assay before 7 days of incubation (27). In our experiments, incubation of leukemic cells in the semisolid bottom layer was extended for 11 days and sometimes even longer (24th day with BI-CML cells) before the upper layers were plated. Nucleated cells ($10^6$/dish) of CML or normal bone marrow cells were used as the test CFU(c) cells in the upper layer. In some of our experiments, we extended the incubation period before counting up to Day 11.

Colonies were counted by inverted microscope, the proper adjustment of which allowed us to determine the level of agar where colonies are located. Colonies were defined as 40 or more cells, and clusters were defined as less than 40 cells/aggregate. The ratio of macrophage and granulocyte CFU(c) colonies was determined. For this purpose, macrophage and granulocyte colonies were counted separately. Some of them were picked up and stained (for methods, see Ref. 27).

**RESULTS**

In all cases of AML and in the cases with APL, AMML, and BI-CML cells treated with $10^{-7}$ to $10^{-9}$ M TPA, transformation to MMCs occurred. Cells attached to the bottom of the plastic flasks acquired the appearance of MMC with thin short and long pseudopods.

MMC from normal bone marrow, CML, and BI-CML marrow can usually be detached only after prolonged and energetic effort; HL-60 cells and AML cells could be detached after simple shaking of flasks. In ALL, treated and untreated cells looked similar. There were no macrophage-like changes in the
lymphoid cells treated with TPA. The only difference between control and $10^{-8}$ M TPA-treated ALL cells was that the latter formed larger clusters of cells.

The experiments studying CSA production from TPA-treated HL-60 leukemic cells are presented in Table 1. The 4 experiments gave similar results. HL-60 cells treated with $10^{-8}$ M TPA used as feeder layer induced CFU(c) colony formation in normal and CML bone marrow.

Untreated HL-60 cells produce no CSA. Concentrations of TPA as small as $10^{-11}$ M are not enough for induction of CSA activity in HL-60 cells.

TPA ($10^{-8}$ M) incubated in culture medium with 20% fetal calf serum without cells for 2, 3, 4, 5, and 6 days and used as feeder layer in CFU(c) assay gave completely negative results. Neither CFU(c) colonies of normal nor CML bone marrow appeared. TPA ($10^{-8}$ M) itself added to the bottom layer agar does not induce CFU(c) colony formation (not presented in tables).

**DISCUSSION**

The experiments presented above confirm and extend our (44, 45) and other (17, 33) previous data on TPA-induced transformation of normal and leukemic myeloid cells to MMCs. Tables 2 and 3 depict the results of TPA treatment of cells from 2 patients with BI-CML and Table 4 the results of experiments with AML cells. It is evident that there is intensive induction of CSA in TPA-treated cells. Also evident is that only prolonged maintenance of the TPA-treated cells in agar gives intense CSA production. The ratio of granulocyte and macrophage CFU(c) colonies and clusters induced with CSA from TPA-treated AML cells was similar when induced with routine blood Buffy coat feeder layer with one exception (see Table 2).

The last experiment (Table 5) indicates that AML blasts treated for only 40 min with $10^{-8}$ M TPA transformed in the next 72 hr to MMCs and produced active CSA.

### Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dose (M)</th>
<th>Time (days)</th>
<th>Incubation in agar (days)</th>
<th>No. of colonies/clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$10^{-8}$</td>
<td>14</td>
<td>Before upper layer plated</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>After upper layer plated</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>$10^{-8}$</td>
<td>8</td>
<td>Normal WBC feeder layer (control)</td>
<td>0/0</td>
</tr>
<tr>
<td>3</td>
<td>$10^{-8}$</td>
<td>8</td>
<td>Normal WBC feeder layer (control)</td>
<td>0/0</td>
</tr>
<tr>
<td>4</td>
<td>$10^{-8}$</td>
<td>8</td>
<td>Normal WBC feeder layer (control)</td>
<td>0/0</td>
</tr>
</tbody>
</table>

*In Experiments 1 and 3 normal bone marrow and in Experiments 2 and 4 CML bone marrow were tested in upper layer.*

### Table 2

**CSA of cells of Patient TH with BI-CML treated with TPA**

Blood Buffy coat cells, these cells depleted of adherent cells, and bone marrow Buffy coat cells were cultured for 3 days in liquid culture with and without $10^{-8}$ M TPA. The cells were then mixed with semisolid agar for feeder layers and incubated from 10 to 23 days. Afterwards, $10^8$ CML bone marrow cells were added to the upper layer. Nine to 23 days later, CFU(c) colonies and clusters were counted.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell specimen</th>
<th>TPA treatment</th>
<th>Incubation time in agar (days)</th>
<th>Experimental feeder layer</th>
<th>Routine normal BM feeder layer</th>
<th>No. of colonies/clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BC</td>
<td>-</td>
<td>10</td>
<td>0/0</td>
<td>78/682</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>BC-ACD</td>
<td>-</td>
<td>10</td>
<td>0/0</td>
<td>78/682</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>BC</td>
<td>+</td>
<td>10</td>
<td>0.5/130</td>
<td>78/682</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>BC-ACD</td>
<td>+</td>
<td>10</td>
<td>5/172</td>
<td>78/682</td>
<td>0/0</td>
</tr>
<tr>
<td>2</td>
<td>BC</td>
<td>-</td>
<td>23</td>
<td>0/0</td>
<td>78/682</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>BC-ACD</td>
<td>-</td>
<td>23</td>
<td>125/355</td>
<td>78/682</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>BC</td>
<td>+</td>
<td>23</td>
<td>56/400</td>
<td>78/682</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>BC-ACD</td>
<td>+</td>
<td>23</td>
<td>219/236</td>
<td>78/682</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>-</td>
<td>23</td>
<td>0/0</td>
<td>78/682</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>BM</td>
<td>+</td>
<td>23</td>
<td>219/236</td>
<td>78/682</td>
<td>0/0</td>
</tr>
</tbody>
</table>

*BC, blood Buffy coat cells, 85% blasts; BC-ACD, BC depleted of adherent cells by 2 hr of plating in Petri dishes; BM, bone marrow Buffy coat cells, 85% blasts and promyelocytes.*

### Table 3

**CSA of cells of Patient V with BI-CML treated with TPA**

Blood Buffy coat cells were incubated 96 hr in liquid cultures with $10^{-8}$ M TPA or without it. They were then placed into semisolid agar as feeder layers and incubated for different periods of time. Afterwards, $10^8$ normal or CML bone marrow nucleated cells were added as upper layer. CFU(c) colony and cluster formation was studied.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>TPA treatment</th>
<th>Incubation time in agar (days)</th>
<th>BM type used for challenge</th>
<th>Experimental feeder layer</th>
<th>Routine normal BC feeder layer</th>
<th>No. of colonies/clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>7</td>
<td>N</td>
<td>16/18</td>
<td>46/33</td>
<td>0/0</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>7</td>
<td>CML</td>
<td>4/23</td>
<td>19/49</td>
<td>0/0</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>21</td>
<td>CML</td>
<td>384/1000</td>
<td>&gt;500</td>
<td>0/0</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>24</td>
<td>N</td>
<td>38/42</td>
<td>30/82</td>
<td>0/0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>24</td>
<td>CML</td>
<td>359/494</td>
<td>160/700</td>
<td>0/0</td>
</tr>
</tbody>
</table>

*BM, bone marrow Buffy coat cells; BC, blood Buffy coat cells, approximately 60% blasts; N, normal.*
The degree of adherence induced is difficult to quantitate but qualitatively differed in the different types of leukemia examined.

TPA in different concentrations can be used as a probe in the study of cells of the hematopoietic system. Thus, 10^{-7} to 10^{-8} M TPA fully inhibited human CFU(c) cells in liquid culture and in semisolid agar (44). These concentrations of TPA induced MMC transformation of leukemic and normal myeloid cells in liquid cultures. At the same time, lymphocytes and ALL cells do not undergo MMC transformation.

Experiments presented above give convincing evidence that, when TPA induces human AML cells (AML, APL, and BI-CML blasts) to MMC transformation, it also induced them to produce macrophage-granulocyte colony-stimulating factor. This hormone-like factor is a typical product of cells of normal monocyte-macrophage lineage of differentiation.

It has also been shown elsewhere that untreated blood buffy coats from AML patients themselves do not possess any CSA (10, 11). This is also confirmed in these experiments (see TPA-untreated groups in each table). Feeder layers of leukemic myeloid cells transformed to MMCs are an active source of CSA and can be compared to feeder layers from normal human buffy coat cells. Continuous exposure to TPA is unnecessary, since AML cells treated 40 min with TPA and washed also transform to MMCs and produce CSA (Table 5). Even without washing, TPA is very soon not detectable in cell cultures (20). Very short time (hr) of presence of TPA in tissue culture (20) demonstrates that TPA has no possible effect by itself. In our system, cells were cultivated 14 days in liquid cultures plus 7 days in semisolid agar or 4 days in liquid culture and 24 days in semisolid agar before the upper layer was added. Longer preincubation was best in respect to CSA production. The inductive role of agar has been eliminated by control groups (found in each experiment) in which TPA-untreated leukemic cells did not produce CSA after incubation in agar.

Several controls showed that CSA is produced by the leukemic blasts treated with TPA themselves but not by some cellular minority present in leukemic blood and bone marrow buffy coats possibly activated by TPA. First of all, such cells are entirely absent in the HL-60 line. HL-60 is easily inducible by 10^{-8} M TPA to produce CSA. Secondly, depletion of plastic adherent cells from the patients’ buffy coats has no definite influence on CSA production induced by TPA (sometimes increased, sometimes diminished). Special controls show that lymphocyte-enriched buffy coat or ALL blasts treated with 10^{-8} M TPA did not produce any CSA. Others have shown in mice that TPA stimulates CFU(c) colony formation without addition of exogenous CSA (5, 41). In their experiments, the possibility...
exists that some cells of the myeloid compartment used as CFU(c) source can be induced by TPA to elaborate CSA which then acts on the CFU(c) cells of the same marrow. Greenberger et al. (12) presented evidence on TPA stimulation of CSA production in mouse bone marrow liquid cultures.

In the described phenomenon of CSA induction in human myeloid cells, TPA functions like a tropic hormone. TPA possesses several other hormone-like functions. It competes with epidermal growth factor for receptors and shares some functions with this hormone (6, 18, 19, 21, 30). Quite recently, an insulin-tropic function of TPA has been shown (25). TPA induces synthesis of some biologically active molecules, plasminogen activator (48), prostaglandins (13), esterases (13, 26), ornithine decarboxylase (29), as well as enhancement of viral gene expression (3), synthesis of viruses (1), and other cellular changes (for review, see Ref. 47). Quite recently, Sundar et al. (42) showed induction by TPA of lymphocyte cellular changes (for review, see Ref. 47). Quite recently, ef al. (12) presented evidence on TPA stimulation of CSA and high phagocytosing activity could conceivably protect approach to CI-CML. Leukopheresed leukemic cells could be monocyte-macrophage lineage. It offers a therapeutic ap

duces synthesis of some biologically active molecules, plas-


ditions with this hormone (6, 18, 19, 21, 30). Quite recently, an
epidermal growth factor for receptors and shares some func-

myeloid cells to MMCs are obscure. The occurrence of trans-

suppressing terminal differentiation (31, 36, 49, 50) or inducing

proliferation of cells of human myeloid leukemia in culture. In

mutagenic factor. Koeffler (16) found that TPA enhanced clonal

cloning of normal "mast" cells in tissue

exists that some cells of the myeloid compartment used as

REFERENCES


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The skillful technical assistance of Carol Vergara and Phyllis Mann is gratefully acknowledged.
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