Prevention of Phorbol Ester Receptor Down Modulation in Human Myeloblastic Leukemia ML-1 Cells by Differentiation-stimulating Serum Components

Hiroshi Sakagami, Rosemary Hromchak, and Alexander Bloch

ABSTRACT

The phorbol esters 12-O-tetradecanoylphorbol-13-acetate, phorbol 12,13-didecanoate, phorbol 12,13-dibutyrate (PDB), and phorbol 12,13-dibenzoate were found to compete with [20-3H]-PDB binding to human myeloblastic leukemia ML-1 cells in approximate proportion to their differentiation-inducing capacity. Fetal bovine serum decreased the down modulation of phorbol ester receptor sites on these cells and increased PDB-induced differentiation. These two activities coeluted upon chromatography of fetal bovine serum on a Sephadex G-150 column. A partially purified fraction from pokeweed mitogen-stimulated human leukocyte-conditioned medium which effectively induced ML-1 cell differentiation also prevented the down modulation of PDB receptors. As indicated by Scatchard analysis, prevention of down modulation was due to stabilization of the number of binding sites rather than to a change in receptor affinity. In view of the previously observed modulation of growth factor binding by phorbol esters, the currently described alteration of phorbol ester receptor activity by differentiation-inducing factors implies an interaction between growth and differentiation factors in receptor modulation.

INTRODUCTION

Some tumor-promoting phorbol diesters have proven to be active inducers of leukemic cell differentiation. Among them is TPA\(^2\), which causes various human leukemia cell lines to mature to more differentiated stages (1, 6,14, 19–21, 23, 31, 33). Using the less lipophilic phorbol derivative [20-3H]PDB (12), evidence has been adduced demonstrating that binding of phorbol esters to specific receptors is an initial step in the physiological action of these compounds (3–5, 7, 9, 17, 27).

Down modulation of phorbol ester receptors, meaning the loss of binding activity due to the decline in number or affinity of specific receptors (10,11), has been reported in numerous assay systems (9, 24, 27, 29, 30). As part of our studies on the mechanisms of drug-induced cell differentiation, we have investigated the interactive effect that phorbol esters and specific serum components exert on the stability of phorbol ester receptors and on the in vitro differentiation of human myeloblastic leukemia ML-1 cells (2, 31, 32). This paper reports the results of this study.

MATERIALS AND METHODS

Materials. [20-3H]PDB (specific activity, 12.2 Ci/mmol) and unlabeled PDB, purchased from Life Systems (Newton, MA), were dissolved in ethanol at concentrations of 8 \(\mu\)M or 2 \(\mu\)M, respectively. TPA was furnished by Chemicals for Cancer Research, Inc., Eden Prairie, MN, and phorbol 12,13-dibenzoate, phorbol 12,13-didecanoate, 4-O-methyl-TPA, phorbol 12,13,20-triacetate, and \(\alpha\)- and \(\beta\)-phorbol were obtained from Sigma Chemical Co., St. Louis, MO. These compounds were dissolved in dimethyl sulfoxide (Sigma) at concentrations of 100 \(\mu\)g/ml and were stored at \(-20^\circ\)C. RPMI Medium 1640 (18), FBS, and calf serum were purchased from Grand Island Biological Co., Grand Island, NY. Human serum from healthy donors was provided by the RPMI blood bank.

Cell Culture. The human myeloblastic leukemia ML-1 cells were cultured in RPMI Medium 1640 supplemented with 10% heat-inactivated FBS, as described previously (31).

Partial Purification of Differentiation Factor. Human peripheral blood mononuclear cells were obtained from heparinized buffy coat by centrifugation on Ficoll-Hypaque, as described previously (32). The mononuclear cells were suspended at 5 \times 10^6 cells/ml in RPMI Medium 1640, free of FBS but containing pokeweed mitogen (1 \(\mu\)g/ml) and were incubated at 37\(^\circ\)C for 48 hr. After removal of cells and debris by centrifugation at 400 \(\times\) g for 10 min, the supernatants were filtered through a 0.45-\(\mu\)m Millipore filter. The filtrate (400 ml) was concentrated to 5 ml in a Micro-pro-di-con concentrator (Biomolecular Dynamics, Beaverton, OR) using PBS as the dialysis solution. The concentrate was applied to a 2.7-\(\times\)37-cm column of Sephadex G-150, equilibrated with PBS, and eluted with PBS, 2-ml fractions being collected. Fractions 32 to 42, which effectively increase cell differentiation as measured by acid phosphatase activity, were pooled (Chart 8A).

[3H]PDB-binding Assay. ML-1 cells (1 \times 10^6 cells) were washed twice with 10-ml aliquots of serum-free RPMI Medium 1640 and were suspended in 0.2 ml of this medium in borosilicate disposable culture tubes (Fisher). Fifty \(\mu\)l of RPMI Medium 1640 containing 115 \(\mu\)M [3H]PDB (1.4 \(\mu\)Ci/ml), other phorbol esters, and serum at the concentrations indicated in the figures were added, and incubation was carried out in an ice bath or at 37\(^\circ\). The reactions were terminated by adding 1.5 ml of ice-cold PBS. The supernatant solutions obtained after centrifugation at 800 \(\times\) g for 15 min were carefully removed by aspiration, and the pellet cells were washed twice with 1.5 ml of ice-cold PBS to remove free PDB. At 0\(^\circ\), less than 5% of the bound radioactivity was released by repeated washing (data not shown). The washed cells were dissolved at 37\(^\circ\) in 0.5 ml of 1% sodium dodecyl sulfate, and the lysates were transferred to scintillation vials containing 5 ml of ACS II scintillation fluid (Amersham). Radioactivity was counted at 40% efficiency in a Packard Tri-Carb Model 3330 spectrometer. Nonspecific binding, determined in the presence of 30 \(\mu\)M unlabeled PDB, was subtracted from total binding to obtain specific binding.

Quantitation of PDB Degradation. ML-1 cells (3 \times 10^6 cells/ml) were incubated for 72 hr with 23 \(\mu\)M [3H]PDB in RPMI Medium 1640. After centrifugation, 50-\(\mu\)l aliquots of the supernatant solution were applied to Silica Gel 60F254 thin-layer plates and were chromatographed in toluene:acetone (3:1), together with authentic TPA, PDB, and phorbol. In this solvent system, these markers had R\(_f\) values of 0.33, 0.25, and 0, respectively, as visualized under UV. To determine the location of radio-
and D) that, in the presence of FBS, the number of binding sites for the ligand, down modulation was decreased by the presence of concentrations and appeared to be saturable, regardless of the presence or absence of FBS. However, at any concentration of serum, binding to intact ML-1 cells increased with increased ligand concentration. At 1 hr after incubation, [3H]PDB binding was stabilized by serum concentrations as low as 0.03%, reaching a maximum within 15 min (Chart 2A). Thereafter, specific binding decreased by approximately 50% at 60 min ("down modulation") (9, 24, 27, 29, 30). This decrease was not due to a decrease in cell viability. Addition of 10% FBS to the incubation medium held down modulation to approximately 16%. With different lots of serum (Chart 2B, C, and D), maximal initial binding was somewhat higher, but the reduction of down modulation in the presence of serum was similar. At 0°, PDB binding was considerably slower, and down modulation did not occur (Chart 2D) confirming previous findings (9, 24, 27, 29, 30). At this temperature, maximal binding in the presence of 10% FBS was achieved within 3 hr, whereas in its absence, approximately 6 hr were required to obtain the same result.

As shown in Chart 3, the extent to which down modulation was prevented was found to be a function of the serum concentration. At 1 hr after incubation, [3H]PDB binding was stabilized by serum concentrations as low as 0.03%, reaching a maximum at 20% serum. Similar results were obtained with serum that had been extensively dialyzed, and the stabilizing effect appears, thus, to be the result of a high-molecular-weight component(s).

As shown in Chart 4, A and B, the extent of specific [3H]PDB binding to intact ML-1 cells increased with increased ligand concentrations and appeared to be saturable, regardless of the presence or absence of FBS. However, at any concentration of the ligand, down modulation was decreased by the presence of FBS. Scatchard analysis (25) of the data showed (Chart 4, C and D) that, in the presence of FBS, the number of binding sites per cell is not markedly changed during the 60-min incubation period at 37° (1.37 x 10^5 → 1.40 x 10^5), whereas in the absence of FBS, a significant decrease occurs (1.31 x 10^5 → 1.01 x 10^5).

Enhancement of Phorbol Ester-induced ML-1 Cell Differentiation by FBS. Chart 5 shows that the extent of TPA-induced differentiation, assayed by morphological change (Chart 5A) and by increased acid phosphatase activity (Chart 5B), also depends on the serum concentration. Some increase in TPA or PDB-induced cell maturation was observed at FBS concentrations

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**RESULTS**

Relationship between Specific Binding of Phorbol Esters and Induction of ML-1 Cell Differentiation. We have reported previously that, upon treatment with TPA, ML-1 human myeloblastic leukemia cells differentiate to morphologically and functionally mature macrophage-like cells (31). Since binding of inducers is considered an initial event in their action, the relative affinity with which a series of phorbol derivatives bind to ML-1 cells was compared with their ability to induce the differentiation of these cells. As shown in Chart 1A, TPA displayed the greatest binding affinity, whereas phorbol dibenzoate, PDB, and phorbol dibenzate were less effectively bound. 4-O-Methyl TPA, α-phorbol, β-phorbol, and phorbol triacetate bound only weakly.

The binding affinity of these agents was paralleled by their capacity to induce the differentiation of the ML-1 cells, as measured by decreased proliferation (Chart 1B), morphological change (Chart 1C), and increased acid phosphatase activity (Chart 1D).

Enhancement of Specific [3H]PDB Binding by FBS. When ML-1 cells were incubated at 37° with 23 nm [3H]PDB in the absence of FBS, specific binding occurred rapidly and reached a maximum within 15 min (Chart 2A). Thereafter, specific binding decreased by approximately 50% at 60 min ("down modulation") (9, 24, 27, 29, 30). This decrease was not due to a decrease in cell viability. Addition of 10% FBS to the incubation medium held down modulation to approximately 16%. With different lots of serum (Chart 2B, C, and D), maximal initial binding was somewhat higher, but the reduction of down modulation in the presence of serum was similar. At 0°, PDB binding was considerably slower, and down modulation did not occur (Chart 2D) confirming previous findings (9, 24, 27, 29, 30). At this temperature, maximal binding in the presence of 10% FBS was achieved within 3 hr, whereas in its absence, approximately 6 hr were required to obtain the same result.

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Chart 2. Effect of FBS on specific \[^{3}\text{H}\]PDB binding and down regulation at 37° (A to C), and at 0° (D). ML-1 cells (1 \times 10^6) were incubated with 23 nM \[^{3}\text{H}\]PDB for the indicated periods of time in the presence of 0% (O) or 10% (•) FBS. Different lots of FBS (a, b, and c) were used to determine the data in Chart 2, A, B, and C. The data in Chart 2D were determined with Lot a. All values were corrected for nonspecific binding, which amounted to 5 to 10% of total binding, irrespective of the presence or absence of FBS. Points, mean of 3 separate assays; bars, S.D.

Chart 3. Effect of serum concentration on specific \[^{3}\text{H}\]PDB binding. ML-1 cells (1 \times 10^6) were incubated for 1 hr at 37° with 23 nM \[^{3}\text{H}\]PDB in the absence (control) or presence of various concentrations of undialyzed (O) or dialyzed (•) FBS. FBS was dialyzed against RPMI Medium 1640. Points, mean obtained from 3 different experiments; bars, S.D. Scatchard plots of the data in A and B, are shown in C and D, respectively, the lines having been fitted by linear regression.

Chart 4. Specific \[^{3}\text{H}\]PDB binding as a function of ligand concentration in the presence (O) or absence (•) of FBS. ML-1 cells (1 \times 10^6) were incubated at 37° for 15 min (A) or 60 min (B) with increasing concentrations of \[^{3}\text{H}\]PDB to determine total binding, or together with 40 \mu M unlabeled PDB to determine nonspecific binding. Specific binding was determined by subtracting the nonspecific binding at each \[^{3}\text{H}\]PDB concentration from the corresponding total value. Points, mean obtained from 3 different experiments; bars, S.D. Scatchard plots of the data in A and B, are shown in C and D, respectively, the lines having been fitted by linear regression.

equal to or greater than 0.3%, indicating a positive correlation between binding and induction of differentiation. ML-1 cells incubated with 0 to 10% FBS alone, did not show a significant increase in the number of differentiated cells. Chart 5, C and D, shows that the extent of PDB-induced differentiation in the absence of FBS was only about 20% of that observed in the presence of 10% FBS (maximum differentiation), even when PDB concentration was elevated up to 5 \times 10^{-8} M. Similarly, 5 \times 10^{-8} M TPA induced only about 30% of maximum differentiation when FBS was omitted from incubation medium (data not shown). Incubation with high concentrations of phorbol esters (>1 \times 10^{-7} M PDB, >1 \times 10^{-8} M TPA) caused extensive cell kill.

Thin-layer chromatography revealed that at least 93% of \[^{3}\text{H}\]-PDB was recovered unchanged after incubation for 72 hr, regardless of the presence or absence of serum, suggesting that phorbol ester degradation is not a significant determinant of the observed differences in down regulation. In fact, the absence of TPA-degrading enzymes from FBS has been noted previously (13).

The contribution which the presence of FBS makes to receptor stabilization and to differentiation is also demonstrated in Chart
Table 1

<table>
<thead>
<tr>
<th>Additions to binding medium</th>
<th>Concentration</th>
<th>Specific [3H]PDB binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(cpm/10^6 cells)</td>
<td>% of increase</td>
</tr>
<tr>
<td>No addition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PWM-CM (Lot a)</td>
<td>1%</td>
<td>1054 ± 82^a</td>
</tr>
<tr>
<td></td>
<td>3%</td>
<td>1316 ± 114</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>1464 ± 126</td>
</tr>
<tr>
<td>No addition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partially purified fraction</td>
<td>0.015 A_{280}</td>
<td>1013 ± 40</td>
</tr>
<tr>
<td></td>
<td>0.019 A_{280}</td>
<td>1334 ± 63</td>
</tr>
</tbody>
</table>

^a Mean ± S.E. from 3 independent experiments.

^b PWM-CM, pokeweed mitogen-stimulated human leukocyte-conditioned medium.

The possibility that nonspecific serum proteins, such as serum albumin, act as stabilizing donors of the ligand to the receptor (16) was examined by adding 0.5 and 5 mg bovine serum albumin/ml to the assay mixture in place of FBS. In both instances, down modulation was spared by only 6%. Also, human serum which contains a multitude of proteins but has only low levels of differentiation-inducing capacity, did not prevent down modulation effectively (Chart 6). Further, nonspecific binding of [3H]PDB was not significantly affected by FBS averaging 153 ±
DISCUSSION

The present report indicates that the biological action of phorbol esters in human myeloblastic leukemia cells, as in other cell systems (5, 7, 9, 27), is mediated by their binding to cellular receptors. This conclusion is based on the observation (Chart 1) that the extent to which phorbol esters induce ML-1 cell differentiation is a function of their binding affinity.

Treatment of the cells with FBS or with a differentiation-stimulating component from leukocyte-conditioned medium leads to decreased down modulation of the phorbol receptors and to an increase in differentiation-inducing activity (Chart 6; Table 1). FBS contains both GFs and DFs, with GF activity predominating. When DFs are added, GF activity is decreased as a function of DF concentration (2, 32), and cell differentiation is stimulated. A similar mechanism may be applicable to phorbol ester-induced cell differentiation. Such esters can interfere with GF binding (15, 26), and the effect of DFs may thereby become enhanced and differentiation may be stimulated. This interpretation is supported by the observation that, in the presence of FBS, TPA is a very effective inducer of ML-1 cell differentiation, whereas in its absence, the ester causes only limited differentiation (Chart 5).

The data presented in this paper point to serum factors as possible modulators of phorbol ester receptor activity, in keeping with the notion (10) that this activity may depend on the cellular environment.

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REFERENCES

17. Rovera, G., Sontak, D., and Damsky, C. Human promyelocytic leukemia cells...


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