Bryostatin 1 Activates Protein Kinase C and Induces Monocytic Differentiation of HL-60 Cells

By Richard M. Stone, Eric Sariban, George R. Pettit, and Donald W. Kufe

Phorbol esters induce the human HL-60 promyelocytic cell line to differentiate along a monocytic pathway. This induction of differentiation may involve phorbol ester-induced activation of the phospholipid-and calcium-dependent protein kinase C. Bryostatin 1, a macrocyclic lactone, has been shown to compete with phorbol esters for binding to protein kinase C. We have confirmed that bryostatin 1 translocates activity of protein kinase C from the cytosolic to membrane fractions of HL-60 cells. The present results also demonstrate that bryostatin 1 (10 nmol/L) induces monocytic differentiation of HL-60 cells as determined by adherence, growth inhibition, appearance of monocyte cell surface antigens, and \( \alpha \)-naphthyl acetate esterase staining. Furthermore, bryostatin 1 (10 nmol/L) downregulated c-myc expression and induced c-fos, c-fms, and tumor necrosis factor (TNF) transcripts. These changes in gene expression induced by bryostatin 1 are similar to those associated with phorbol ester-induced monocytic differentiation of HL-60 cells. In contrast, exposure to a higher concentration of bryostatin 1 (100 nmol/L) had less of an effect on growth inhibition of HL-60 cells and changes in gene expression. Moreover, 100 nmol/L bryostatin 1 antagonized the cytoplastic effects and adherence induced by phorbol esters. Our results thus suggest that bryostatin 1 activates HL-60 cell protein kinase C and that this effect is associated with induction of monocytic differentiation.

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From the Laboratory of Clinical Pharmacology, Dana-Farber Cancer Institute, Harvard Medical School, and the Cancer Research Institute, Department of Chemistry, Arizona State University, Tempe.

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Address reprint requests to Richard M. Stone, MD, Dana-Farber Cancer Institute, 44 Binney St, Boston, MA 02115.

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in fact, an inducer of HL-60 differentiation as assessed by inhibition of growth, adherence, cytochemistry, immunophenotype, and changes in protooncogene expression.

**MATERIALS AND METHODS**

**Cell culture.** HL-60 cells, obtained at passage 13 from American Type Culture Collection (Rockville, MD), were grown in RPMI 1640 medium (Hazelton Laboratories, Vienna, VA) containing 4 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mmol/L nonessential amino acids, and 10 mmol/L sodium pyruvate (GIBCO, Grand Island, NY) in 15% heat-inactivated fetal bovine serum (Hazelton) at a density of 2 x 10⁶/L/mL in a 5% CO₂ humidified atmosphere at 37°C. Viable cells were determined by trypan blue exclusion. HLA typing and p53 deletion on Southern blotting verified that the cells were of HL-60 origin. TPA (Sigma Chemical Co, St Louis) was dissolved in 1% acetone at 3.2 x 10⁻⁶ mol/L and then further diluted to 1.6 x 10⁻⁶ in culture medium. Bryostatin 1 was dissolved in dimethyl sulfoxide at 5 x 10⁻⁴ mol/L and then diluted to 10⁻⁷ or 10⁻⁸ mol/L in the culture medium.

**Induction of monocytic differentiation.** Cell adhesion was determined by calculating the percentage of nonadhered cells compared with the total cells (after adhered cells were scraped from plastic culture flasks).

Cyto centrifuge smears of culture cells were examined after one or two days of treatment with TPA or bryostatin 1 for NSE staining and nitro blue tetrazolium (NBT) reduction.

Monoclonal antibodies MOL, My4 and ID3 were used as previously described in the laboratory of Dr James Griffin (Division of Tumor Immunology, Dana-Farber Cancer Institute). MOL and My4 react with the cell surface antigens of monocytes, whereas ID3 reacts with an antigen present on mature granulocytes. Results are expressed as the percentage of fluorescent cells compared with a control antibody and are based on at least two determinations.

**RNA extraction and hybridization.** Total cellular RNA was purified by the guanidine thiocyanate–cesium chloride method and analyzed by gel electrophoresis through 1% agarose-formaldehyde gels; this was followed by Northern blot transfer to nitrocellulose.

Hybridization conditions were as previously described.

Probes included the 1.6-kilobase (kb) ClaI/EcoRI fragment of the human c-myc 3' exon purified from the PM C41-3 RC plasmid, the 2.7-kb Xhol/NcoI fragment of the p-cf os (human)-1 plasmid, the 2.0-kb PstI fragment of the chicken β-actin gene purified from the pAl plasmid, the 1.3-kb PstI fragment of the v-fms gene isolated from the plasmid pSM3, and the pE4 plasmid containing a 1.1-kb PstI fragment of the TNF cDNA.

**Protein kinase C activity.** Cells (4 x 10⁶) were washed twice with ice-cold phosphate-buffered saline (Ca²⁺ and Mg²⁺ free) containing 2 mmol/L dithiothreitol and lysed by ten aspirations through a 25-needle in 400 µL of column buffer (20 mmol/L Tris-HCl, pH 7.5, 2 mmol/L EDTA, 2 mmol/L dithiothreitol) containing 25 µg leupeptin. Homogenates were sedimented at 12,000 g for 15 minutes to yield cytosol and particulate fractions. The pellet was suspended in column buffer containing 1% Triton X-100, sonicated for 15 seconds, and incubated for one hour on ice to solubilize membrane-bound protein kinase C. Cytosol and particulate fractions were partially purified on 0.4 mL DE52 preequilibrated cellulose columns at 4°C by elution with 1.0 mL of column buffer containing 80 mmol/L NaCl. The enzyme was assayed by a modification of a previously described procedure measuring the incorporation of ³²P from [γ³²P]adenosine triphosphate (ATP) (specific activity, 3,000 Ci/mmol; Amersham Corp, Arlington Heights, IL) into histone H11-S histone. The reaction mixture (250 µL) contained 2.5 nmol [γ³²P]ATP (10⁻⁶ cpm), 20 mmol/L Tris/Cl, pH 7.5, 6 mmol/L magnesium acetate, 1.5 mmol/L CaCl₂, 100 µg/mL phosphatidyserine, 10 µg/mL diolein, and 400 µg/mL histone H11-S. The reaction was initiated by the addition of 100 µL enzyme (~40 µg protein), incubated at 30°C for ten minutes, and terminated by adding 0.3 mL 25% trichloroacetic acid. Protein kinase C activity is reported as ³²P incorporation per microgram of protein in the presence of 1.5 mmol/L added Ca²⁺, 100 µg/mL phosphatidyserine, and 10 µg/mL diolein minus that measured in the presence of 0.8 mmol/L EDTA, 1 mmol/L ethylene glycol tetraacetic acid, and 100 µg/mL phosphatidyserine. The amount of ³²P incorporated in the presence of the calcium chelators and in the absence of diolein was less than 20% of that obtained in the presence of cofactors. Protein was measured on duplicate samples by the Bradford assay using γ-globulin as a standard.

**RESULTS**

**Cell growth and differentiation.** The induction of terminal HL-60 cell differentiation is associated with growth inhibition. In contrast to TPA (16 nmol/L), which completely inhibits HL-60 cell growth, bryostatin 1 (10 nmol/L) only partially slowed proliferation (Fig 1). A higher concentration of drug (100 nmol/L) had less of a growth-inhibitory effect than that observed for HL-60 cells treated with 10 nmol/L bryostatin 1. Furthermore, when HL-60 cells were exposed to both 16 nmol/L TPA and 100 nmol/L bryostatin 1, growth was similar to that obtained with bryostatin 1 alone. Thus bryostatin 1 appears to antagonize the growth-inhibitory effects of TPA.

The partial inhibition of HL-60 growth by bryostatin 1 was associated with phenotypic changes consistent with monocytic differentiation. These changes included an increase in NSE staining and induction of monocytic cell surface markers MOL and My4 (Table 1). In contrast, ID3 cell surface antigen expression was not detectably increased after bryostatin 1 treatment (data not shown), thus suggesting that the effects of this agent were limited to the induction of monocytic and not granulocytic differentiation. Although 100 nmol/L bryostatin 1 had less of an effect on adherence than did 10 nmol/L bryostatin 1, there was no detectable difference at these concentrations in terms of the induction of monocytic cell surface markers. Furthermore, bryostatin 1 (100...
Fig 2. Northern blot analysis of total RNA (20 μg/lane) isolated at the indicated times after bryostatin 1 (10 nmol/L) treatment of HL-60 cells. The lane labeled HL-60 represents RNA from untreated cells.

Table 1. HL-60 Cell Differentiation

| Treatment                          | Cell Adhesion | NSE† Staining | Cell Surface Markers†
<table>
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<tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mol</td>
</tr>
<tr>
<td>Untreated</td>
<td>4.0 ± 2.1</td>
<td>+</td>
<td>4.5 ± 1.4</td>
</tr>
<tr>
<td>Bryostatin 1, 10 nmol/L</td>
<td>27.3 ± 5.5</td>
<td>+++</td>
<td>22.0 ± 9.0</td>
</tr>
<tr>
<td>Bryostatin 1, 100 nmol/L</td>
<td>8.7 ± 4.3</td>
<td>++</td>
<td>19.6 ± 7.8</td>
</tr>
<tr>
<td>TPA, 16 nmol/L</td>
<td>78.7 ± 11.3</td>
<td>++++</td>
<td>30.7 ± 2.3</td>
</tr>
<tr>
<td>Bryostatin 1, 100 nmol/L, and TPA, 16 nmol/L</td>
<td>15.3 ± 2.5</td>
<td>+</td>
<td>27.5 ± 4.5</td>
</tr>
</tbody>
</table>

*Measured as described at 36 hours of treatment and expressed as the mean percentage adhered ± SE of three separate experiments. Adhesion with bryostatin 1, 10 nmol/L, TPA, 16 nmol/L, and the combination treatment were each greater than control. Adhesion with 10 nmol/L bryostatin 1 was greater than that for 100 nmol/L bryostatin 1 (P < .05, Student’s t test).

†Based on scoring cytocentrifuged cells treated for 36 hours in at least two separate experiments; scale, + to ++++. Percent positive cells above background after 36 hours of treatment. Values represent the means ± SE of three separate experiments except for the combination treatment, which was from two experiments. All values in treated cells are significantly greater than control values (P < .05, Student’s t test).

nmol/L) antagonized TPA-induced adhesion, whereas there was little if any effect of this agent on TPA-induced expression of the monocyte surface markers (Table 1). Neither TPA nor bryostatin 1 increased NBT staining (data not shown), thus indicating similar statuses in the mature phenotype induced by either agent.

In HL-60 cells treated for three days, 0.01, 0.1, and 1,000 nmol/L bryostatin 1 had no significant effect on either growth or adherence. Furthermore, cell counts at three days in the presence of 100 or 1,000 nmol/L bryostatin 1 were greater than those obtained in the presence of 10 nmol/L bryostatin 1. Adherence after three days was significantly greater than in the control only after treatment with 1 or 10 nmol/L bryostatin 1 (data not shown).

RNA expression. Similar but not identical changes in the levels of c-myc, c-fos, c-fms and TNF transcripts were detected in bryostatin 1–treated cells as compared with that previously observed after treatment with TPA6 (Fig 2). The level of c-myc transcripts was increased at one to three hours but was downregulated after 12 hours. Similar findings were obtained with TPA treatment.6 c-fos Transcripts were detected at 30 minutes after bryostatin 1 treatment, subsequently declined, and were again detectable at 24 hours. This biphasic activation of c-fos expression has not been observed after TPA treatment of HL-60 cells.6 c-fms Transcripts appeared at 12 and 24 hours after bryostatin 1 treatment. Moreover, TNF transcripts were detectable at 30 minutes of bryostatin 1 treatment and declined by 12 hours. These changes in c-fms and TNF expression were similar to those observed in TPA treated cells (Sariban et al6 and unpublished data). Finally, the stability of actin transcripts during bryostatin 1 treatment (Fig 2) implied that the observed changes in gene expression were unrelated to nonspecific effects or to differences in the amount of RNA on the hybridization filters.

Our initial studies demonstrated that 10 nmol/L bryostatin 1 had more pronounced effects on HL-60 growth inhibition and adherence than did a tenfold-higher concentration of drug. Consequently, we compared changes in protooncoprotein expression at both 10 and 100 nmol/L bryostatin 1. The results indicate that treatment with 10 nmol/L bryostatin 1 for 24 hours resulted in a more pronounced downregulation of c-myc expression. Furthermore, 10 nmol/L bryostatin 1

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**Fig 2.** Northern blot analysis of total RNA (20 μg/lane) isolated at the indicated times after bryostatin 1 (10 nmol/L) treatment of HL-60 cells. The lane labeled HL-60 represents RNA from untreated cells.
induced higher levels of c-fos and c-fms transcripts (Fig 3). Thus, the lower concentration of bryostatin 1 was also more effective in inducing changes in protooncogene expression after 24 hours of treatment. However, after 90 minutes of treatment there was little if any difference in c-myc and c-fos mRNA levels (determined by densitometric tracings) at the two concentrations of bryostatin 1.

Protein kinase C activity. Activation of protein kinase C is accompanied by translocation of enzyme activity from the cytosol to membrane fractions. Consequently, protein kinase C activation was monitored by changes in the level of protein kinase C activity in each fraction from treated cells as compared with control cells. The results indicate that 10 nmol/L bryostatin 1 and 16 nmol/L TPA treatment induced a loss of cytosolic activity and a gain in membrane-associated activity relative to control cells (Table 2). The activation of protein kinase C by both bryostatin 1 and TPA occurred as rapidly as five minutes after treatment and was maximal at 30 minutes (data not shown). In contrast, although the percentage of total protein kinase C activity associated with the membrane fraction was similar after treatment at both concentrations, 100 nmol/L bryostatin 1 resulted in a decline in total activity compared with that observed with 10 nmol/L drug. This decline was manifested by a fall in cytosolic protein kinase C activity and the absence of a concomitant rise in membrane activity when compared with untreated cells.

DISCUSSION

The present results confirm the finding that bryostatin 1 activated protein kinase C in intact HL-60 cells. However, although previous studies suggested that this agent is ineffective in inducing HL-60 differentiation, we demonstrated that treatment of these cells with bryostatin 1 leads to phenotypic changes and changes in gene expression that are similar to those associated with TPA-induced monocytic differentiation. The basis for this discrepancy remains unclear, although the HL-60 cell lines used may have differed in their capacity to respond to bryostatin 1. In this regard, HL-60 cell variants have been described that fail to exhibit TPA-induced activation of protein kinase C. Furthermore, although the membrane-associated protein kinase C activity for HL-60 cells (approximately 0.2 pmol/min/µg) is similar to that reported elsewhere, the total activity is lower, thus accounting for a relatively higher amount in the membrane (38% vs 7%). The magnitude of protein kinase C translocation after treatment with bryostatin 1 or phorbol ester in our study is also less than that reported by others. The basis for these discrepancies may be related to the different proteolytic inhibitors used in the studies. Moreover, immunologic localization of protein kinase C was not performed as another approach to demonstrate translocation. However, the induction of HL-60 differentiation by bryostatin 1 is in concert with the recent demonstration that this agent also induces differentiation, as measured by adherence, in the human acute myeloblastic leukemia cell line KG-1. Although both bryostatin 1 and TPA induce activation of protein kinase C and appearance of the HL-60 monocytic phenotype, there are certain differences between these agents in terms of their effects on growth and adherence. These differences might be related to selective effects on

Table 2. Protein Kinase C Activity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytosol (µmol ATP/µg/min)</th>
<th>Membrane (µmol ATP/µg/min)</th>
<th>Total (µmol ATP/µg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.38 ± 0.11</td>
<td>0.23 ± 0.06</td>
<td>0.61 ± 0.16</td>
</tr>
<tr>
<td>Bryostatin</td>
<td></td>
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<td></td>
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<tr>
<td>10 nmol/L</td>
<td>0.17 ± 0.03*</td>
<td>0.29 ± 0.06*</td>
<td>0.47 ± 0.08</td>
</tr>
<tr>
<td>100 nmol/L</td>
<td>0.09 ± 0.04</td>
<td>0.19 ± 0.06</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>TPA, 16 nmol/L</td>
<td>0.29 ± 0.21</td>
<td>0.44 ± 0.17†</td>
<td>0.73 ± 0.38</td>
</tr>
</tbody>
</table>

Cells were treated for 30 minutes with paired untreated controls for each experiment. Results are expressed as pmol ATP/µg/min (mean ± SE) from the indicated (in parentheses) number of experiments compared with control by Student’s paired t test.

*P < .05.
†P < .01.
substrate specificity has been proposed to explain differences in prolactin synthesis and secretion in bryostatin 1- and TPA-treated GH4C1 pituitary cells. Although bryostatin 1 and TPA were agonists for activation of purified protein kinase C, it is possible that the two agents are partial agonists for the activation of the enzyme in intact cells, at least for some substrates. A gene family has been described for the expression of multiple isoenzyme forms of protein kinase C. Thus, bryostatin 1 and TPA could activate distinct members of this family and thereby have different patterns of substrate specificity. In fact, bryostatin 1 has been shown to result in the phosphorylation of specific acidic nuclear envelope proteins that are not phosphorylated in TPA-treated cells. Finally, the differences observed between bryostatin 1 and TPA might also be related to diverse effects of these agents on events unrelated to the activation of protein kinase C.

The present results also demonstrate that 100 nmol/L bryostatin 1 induces fewer growth-inhibitory effects, less adherence, and less in the way of changes in protooncogene expression (at 24 hours of treatment) as compared with a tenfold-lower drug concentration. In contrast, both concentrations of bryostatin 1 resulted in a similar induction of monocyte surface markers. Moreover, treatment with both 10 and 100 nmol/L bryostatin 1 resulted in a similar percentage of protein kinase C activity translocated to the membrane, although the total enzyme activity was lower at the higher concentration. These findings might involve the activation of events at higher but not lower concentrations of bryostatin 1 that inhibit differentiation. Similar biphasic effects have been described for the induction of monocytic differentiation by lipopolysaccharide and glucagon-induced inositol phosphate turnover in hepatocytes. Tenfold-higher concentrations of TPA than those used in the present study are too toxic to HL-60 cells to permit analogous experiments.

In summary, the present results demonstrate that bryostatin 1 induces monocytic differentiation of HL-60 cells. Because bryostatin 1 also activates protein kinase C, this lends support to the importance of this enzyme in the pathway leading to the differentiated phenotype. Other activators of protein kinase C such as teleocidin and 1,2-dioctanoyl glycerol also induce differentiation of HL-60 cells. Bryostatin 1, like other agents that activate protein kinase C, may bypass the physiological receptor-mediated activation of phospholipase C and subsequent phosphoinositide turnover. In this regard, we have recently demonstrated that exogenous phospholipase C also induces HL-60 differentiation. Finally, further studies exploring the role of the bryostatins as differentiation-inducing agents are warranted on the basis of the present findings as well as recent findings that demonstrate that this agent can substitute for granulocyte-macrophage CSF in supporting normal human colony formation in vitro.

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