Functionally Deficient Differentiation of HL-60 Promyelocytic Leukemia Cells Induced by Phorbol Myristate Acetate

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ABSTRACT

The human promyelocytic leukemia cell line HL-60 undergoes terminal myeloid differentiation in vitro in response to a wide variety of chemicals. The tumor promoter phorbol myristate acetate induces these cells to develop macrophage-like morphology, adherence, and enzymatic characteristics. The present study confirms those observations and further documents the induction, by 16 nm phorbol myristate acetate, of 5'-nucleotidase activity, another human macrophage marker enzyme. However, more importantly, functional studies show that phorbol myristate acetate-induced HL-60 cells fail to increase above base line uninduced levels of hexose monophosphate shunt activity, superoxide generation, nitroblue tetrazolium reduction, bacterial ingestion, or complement secretion. These cells therefore possess some macrophage-like properties but do not meet several important functional criteria of macrophage identity.

INTRODUCTION

The study of hematopoiesis has greatly benefited from the in vitro model systems provided by continuous culture cell lines that differentiate in response to various inducing agents. Mouse erythroleukemia (27) and myeloid leukemia (36) cell lines have received extensive study. More recently, Collins et al. (6, 7) have developed a human promyelocytic leukemia cell line, HL-60, that undergoes terminal differentiation in response to some of the same in vitro chemicals that induce maturation of the mouse lines. The most effective of these compounds, polar solvents such as DMSO,3 induce not only morphological maturation to band and polymorphonuclear forms but also functional differentiation. HL-60 cells cultured in 1.3% DMSO generate superoxide, reduce NBT, show chemotactic responsiveness, ingest particles, degranulate, and kill bacteria nearly as well as do normal human granulocytes (8, 15, 30). However, they do not contain the specific granule markers lactoferrin (30) and B18-binding protein (15) or specific granules recognizable by electron microscopy (15).

Recently, Huberman and Callahan (19) reported the induction of terminal differentiation in HL-60 cells by PMA and other related phorbol diesters. These compounds are natural products responsible for tumor promotion by croton oil (18). PMA-induced differentiation appeared to be myeloid on the basis of morphological change and yeast ingestion. However, Rovera et al. (33–35, 40) and Lotem and Sachs (24) have shown that PMA-induced HL-60 cells are "macrophage-like" on the basis of light and electron microscopic morphology, adherence to plastic, increased levels of NADase and α-naphthyl acetate esterase, decreased peroxidase and chloroacetate esterase, and synthesis of acid phosphatase with an isoenzyme pattern typical of monocytes. PMA also induced several characteristics common to both granulocytes and macrophages, including phagocytosis of IgG-coated erythrocytes, increased levels of lysozyme, and decreased levels of myeloperoxidase (24, 33, 35). Both uninduced and induced cells had Fc receptors for IgG (35).

The present study examines a macrophage enzyme marker, 5'-nucleotidase (12), and several functional capacities, including secretion of complement components, ingestion, and respiratory burst activity. The results indicate that PMA-induced HL-60 cells develop the morphological appearance and enzymatic characteristics of macrophages but lack the functional capacities of mature phagocytic cells.

MATERIALS AND METHODS

HL-60 cells, generously provided by Dr. Robert C. Gallo (National Cancer Institute, Bethesda, Md.), were grown as previously described (30) in Roswell Park Memorial Institute Tissue Culture Medium 1640 (M. A. Bioproducts, Walkersville, Md.) with 10% fetal calf serum (M. A. Bioproducts) plus various concentrations of PMA, 4-O-methyl-PMA (Consolidated Midland Chemical Co., Brewster, N. Y.), or DMSO (Fisher Scientific Co., Fair Lawn, N. J.). The DMSO concentrations equalled the solvent carried over from the PMA stock solutions and at most reached only 1/250 the concentration used to produce myeloid differentiation. Cells were counted and media were changed every other day.

Control human peripheral blood monocytes were obtained by sedimentation of citrate-anticoagulated venous blood in 1% Dextran 500 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and centrifugation over Ficoll-Paque (Pharmacia). The interface contained 65 to 85% monocytes, with the remainder being lymphocytes. Macrophages were obtained from human spleens removed at staging laparotomy for Stage I or Stage II Hodgkin's disease. Sections were finely minced and centrifuged over Ficoll-Paque, and adherent cells were separated from the interface by incubation on tissue culture plastic in Roswell Park Memorial Institute Tissue Culture Medium 1640.
with 10% fetal calf serum. Results for monocyte and macrophage preparations were corrected for the percentage of phagocytic cells observed microscopically after Wright-Giemsa staining.

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) activity was measured by the method of Avruch and Wallach (2) using [2-3H]AMP (Amersham-Searle Corp., Arlington, Ill.) as substrate and counting the release of labeled nucleoside.

\( \alpha \)-Naphthyl (nonspecific) esterase activity was determined histochemically (on HL-60 cells and simultaneous fresh human bone marrow controls) as described by Koski et al. (23) using \( \alpha \)-naphthyl butyrate substrate. Myeloperoxidase assays on cell lysates followed a previously described spectrophotometric procedure (30). Results for enzyme determinations are expressed as the means of duplicate or triplicate determinations with less than 10% variability.

Titrations of C2 were performed by hemolytic assay as described by Rapp and Borsos (32). Briefly, media from 48- and 96-hr cell cultures of HL-60 (with or without PMA) and human peripheral blood monocytes were stored frozen at -70°C and then assayed at dilutions of 1:5, 1:15, and 1:45. Normal human serum served as a positive control. In the titration of C2, a constant number of indicator cells was exposed to the test samples. The indicator cells were prepared with sheep erythrocytes, hemolysin, guinea pig Cl, and human C4. The degree of complement-induced hemolysis was determined by spectrophotometric measurement of hemoglobin release. Immunogenic C4 was measured by immunoabsorption from medium in which cells were grown with \([\text{35S}]\)methionine and autoradiography of sodium dodecyl sulfate-polyacrylamide electrophoresis gels of the immunoprecipitate (17). C4 hemolytic activity was determined by a one-step hemolytic assay developed by Atkinson et al. (1). Serial dilutions of culture medium were added to a constant number of indicator cells (sheep erythrocytes coated with a subagglutinating amount of IgG (Cordis Laboratories, Inc., Miami, Fla.) and C4-deficient guinea pig serum. After 1 hr incubation at 37°C, complement-induced hemolysis was read spectrophotometrically and compared to dilutions of known standards. Monocyte culture medium for positive controls was obtained 4 weeks from establishment of human peripheral blood monocyte cultures, grown as described by Einstein et al. (13).

For measurements of ingestion, cultures were grown, and then assays were performed at 96 hr in the same 15-ml plastic tubes (No. 2095, Falcon Plastics, Oxnard, Calif.). Thus, adherent cells were not removed or disrupted for the purpose of the assay. The tubes were centrifuged at 200 x g for 10 min, and the pellet was resuspended in 10 ml Krebs-Ringer phosphate buffer, pH 7.4 (reaching a level to immerse adherent cells that had not spun down), containing \(^{14}C\)-labeled Escherichia coli (5 \times 10\(^3\)/ml) previously opsonized in fresh human serum. Bacteria opsonized in heat-inactivated serum were not ingested in this assay system. Control tubes contained in addition 1 mm N-ethylmaleimide (Sigma Chemical Co., St. Louis, Mo.). After shaking the tubes for 20 min at 37°C, we added 20 ml of cold 1 mm N-ethylmaleimide in 0.154 M NaCl, centrifuged the mixture at 600 \times g for 10 min, and washed the pellet twice in like manner. The final pellet was dissolved in 1 N NaOH, and aliquots were taken for protein determination (25) and scintillation counting. Results were calculated as cpm/mg protein for each tube and expressed (for this and all other measurements) as the mean ± S.E. of at least triplicate determinations.

Measurements of superoxide generation and HMPS activity utilized cells grown in plastic tubes as for the ingestion assay. Mean protein determinations from 3 tubes from each set of culture conditions provided the basis for calculating specific activities. Superoxide production was measured by cytochrome c reduction according to the method of Babior et al. (3) except that opsonized zymosan (6 mg/ml) served as the stimulant. HMPS activity, also stimulated by opsonized zymosan, was measured as the release of \(^{14}CO_2\) from the labeled first carbon of glucose, as described by Stossel et al. (38). NBT slides were prepared as described previously (30) from 22- x 22-mm sterile glass coverslips inserted into 35-mm plastic Petri dish cultures, removed at 96 hr, gently washed with 0.154 M NaCl, and immersed in the reaction mixture. Control slides showed no NBT reduction in the presence of 1 mm N-ethylmaleimide to inhibit the superoxide-generating system (30).

**RESULTS**

HL-60 cells cultured in 16 nm PMA underwent the same macrophage-like morphological, proliferative, and enzymatic changes reported previously (24, 33-35, 40). As shown in Fig. 1, cells incubated in PMA changed from their usual, round, nonadherent state (thin arrow) to adherent cells with either extended pseudopodia (thick arrow) or a broad veil of adherent, granule-excluding cytoplasm (open arrow). Proliferation ceased (as monitored by cell counts), but fewer than 15% of the cells failed to exclude trypan blue. Table 1 presents the enzyme contents of HL-60 cells incubated for 4 days in 16 nm PMA and of uninduced controls. Incubation in PMA induced histochemically detectable \( \alpha \)-naphthyl butyrate (nonspecific) esterase in 75% of HL-60 cells compared to a base line of 3%, and resulted in an 8-fold decrease in myeloperoxidase content. 5'-Nucleotidase activity was undetectable in uninduced HL-60 cells and appeared in PMA-treated cells at a level approximately two-thirds that in human splenic macrophages. None of these changes occurred in HL-60 cells exposed to 16 nm 4-O-methyl-PMA, a far less active phorbol derivative (18).

The functional characteristics of HL-60 cells exposed to 16 nm PMA for 4 days, shown in Table 2, remained the same as those of uninduced cells. Their capacities for HMPS activity, superoxide generation, NBT reduction, ingestion, and secretion of C2 were considerably lower than those of control monocytes and macrophages. Monocytes incubated for 1 hr in 16 nm PMA showed normal function in these assays. Neither HL-60 cells nor human monocyte cultures produced detectable hemolytic or immunogenic C4 (data not shown). Similar results in functional assays were obtained with 3 and 80 nm PMA and with 16 nm 4-O-methyl-PMA as inducing agents.

**DISCUSSION**

Tumor-promoting phorbol esters have attracted a recent resurgence of interest, particularly regarding their effects on *in vitro* cell growth and differentiation. These effects are varied and often paradoxical. PMA can both enhance and inhibit differentiation of mouse erythroleukemia (26), human melanoma (28), and mouse myeloid leukemia (22, 29) cell lines. It
PMA-induced Differentiation of HL-60

Fig. 1. HL-60 cells in PMA. The culture was grown in medium containing 16 nM PMA and photographed by phase-contrast microscopy after 48 hr. Large solid arrow, adherent cell with macrophage-like size, spreading, and pseudopodia; large clear arrow, adherent macrophage-like cell without pseudopodia. Small arrow, nonadherent cell similar in appearance to uninduced HL-60; bar, 10 µm.

also inhibits myogenesis (5), adipocyte conversion of a fibroblast cell line (11), and the differentiation of neuroblastoma cells (20). PMA stimulates macrophage growth and differentiation in normal mouse bone marrow grown in semisolid medium (14, 24, 39) or long-term liquid culture (16). It induces macrophage-like changes in vitro in leukemic cells from some patients with acute myeloid (but not lymphoid) leukemia (31). The human promyelocytic leukemia cell line HL-60, which differentiates into mature granulocytes upon exposure to DMSO and other inducing agents, undergoes macrophage-like terminal differentiation when cultured in 16 to 160 nM PMA (24, 33–35, 40). Cloned murine myeloid leukemia cell lines have shown a similar capacity for bipotential differentiation to granulocyte or macrophage morphology (36).

The present study examined PMA-induced HL-60 cells to determine how well these morphologically macrophage-like cells fit a more complete set of properties defining a macrophage. Cline (4) has suggested as such criteria: (a) size (20- to 80-µm diameter); (b) low or absent proliferative capacity; (c) adherence to glass and certain plastic surfaces; (d) many lysosomal granules and high lysosomal enzyme activities, except for myeloperoxidase; (e) respiratory burst activity; (f) phagocytosis; (g) synthesis of biologically active serum proteins such as endogenous pyrogen and certain complement components. Granulocytes share all but the first and last of these properties but contain considerable myeloperoxidase activity. Human granulocytes and macrophages probably also differ in their display of the plasma membrane marker 5'-nucleotidase. Human neutrophils show no such activity (37), but monocytes develop it over 24 to 48 hr in culture (21). We have found no reports examining its activity in human macrophages harvested after maturation in vivo.

HL-60 cells cultured in PMA meet the first 4 of the above criteria, as described previously (24, 33, 34, 35, 40), confirmed in the present study, and extended by our finding of induction of 5'-nucleotidase activity. However, they show no increase in respiratory burst function (NBT reduction, superoxide generation, and HMPS activity) or ingestion of complement-opsonized bacteria above the low level displayed by control cultures exposed to a low concentration of DMSO (5 ppm) or the inactive phorbol derivative, 4-O-methyl-PMA. A frequent, but not entirely consistent, observation in our ingestion assay was

Table 1

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<th>Enzymatic characteristics of PMA-induced HL-60 cells</th>
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<td>HL-60 cells at Day 4 with or without 16 nM PMA and human splenic macrophages were assayed for enzyme presence (esterase) or activity (myeloperoxidase and 5'-nucleotidase) as described in “Materials and Methods.” Results represent the means of duplicate determinations within 10% of each other.</td>
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<th></th>
<th>Naphthyl butyrate esterase (% of positive cells)</th>
<th>Myeloperoxidase (ΔA_{200}/min/10^6 cells)</th>
<th>5'-Nucleotidase (nmol/min/mg protein)</th>
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<tr>
<td>PMA-induced</td>
<td>75</td>
<td>0.74</td>
<td>0.163</td>
</tr>
<tr>
<td>Uninduced</td>
<td>3</td>
<td>5.92</td>
<td>0.000</td>
</tr>
<tr>
<td>Macrophages</td>
<td>ND&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>0.248</td>
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<sup>a</sup> ND, not determined.
that labeled bacteria adhered more readily to PMA-induced HL-60 cells than to uninduced cells, although the number actively ingested did not differ. This adherence phenomenon may relate to their acquisition of nonspecific "stickiness" (e.g., to glass and plastic) and may explain previous reports of enhanced ingestion based on visual microscopic assays that could not distinguish adherent from ingested particles. Alternatively, ingestion of nonopsonized yeasts by PMA-treated HL-60 cells (19) could represent induction of a receptor-independent mechanism different from the one that we measured with complement-opsonized E. coli. Growth of HL-60 cells in PMA also fails to induce complement (C2 or C4) secretion. These effects of low concentrations of PMA over several days in culture differ entirely from the dramatic stimulation of phagocyte functional differentiation. Nor, in fact, should the cells meet several important criteria of a true macrophage. The PMA possesses some macrophage-like properties but fails to inhibit morphological differentiation in cultured mouse neuroblastoma cells. Thus, the end product of HL-60 differentiation induced by PMA possesses some macrophage-like properties but fails to meet several important criteria of a true macrophage. The system therefore does not seem useful as a model for macrophage functional differentiation. Nor, in fact, should the cells be termed macrophages, for although they share some phenotypic characteristics, they lack the most important functions of mononuclear phagocytes. These studies also reemphasize the need to include functional criteria in addition to morphological and biochemical characterization in the classification of cell lines.

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REFERENCES


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