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Induction of terminal differentiation in human promyelocytic leukemia cells by tumor-promoting agents

(myeloid differentiation/phagocytosis/phorbol diesters)

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ABSTRACT **Human promyelocytic leukemia cells (HL-60) were induced to differentiate into mature cells by the tumor-promoting agent phorbol-12-myristate-13-acetate and other related phorbol diesters. Differentiation was determined by an increase in the percent of myelocytes, metamyelocytes, and other mature myeloid cells as well as by an increase in the percent of phagocytizing cells. Induction of differentiation could be determined after 2 days of treatment with phorbol-12-myristate-13-acetate at a dose as low as 6×10^{-11} M. A correlation was found between reported tumor-promoting activity of a series of phorbol esters and their ability to induce myeloid differentiation and to inhibit cell growth. It is suggested that tumor-promoting agents like chemicals that induce terminal differentiation in these cells, at extremely low concentrations, may be used as a tool in the study of the control of cell growth, cell differentiation, and malignancy in human leukemic cells.**

Etablished cell lines with appropriate markers for cell differentiation offer simple models for study of the control of cell growth and differentiation (1–5). These cell culture systems also allow the identification and study of the mode of action of chemicals that may control cell differentiation (4–11). Phorbol-12-myristate-13-acetate (PMA) and other related phorbol esters (12), which are tumor promoters in a two-stage mouse-skin carcinogenesis system (13), were recently found to inhibit spontaneous and induced cell differentiation in avian myoblasts (14) and in murine erythroleukemia (15, 16), neuroblastoma (17), and adipose cells (18). These experiments suggest that tumor-promoting agents may mimic natural cellular agents that are involved in the regulation of cell growth and differentiation. Therefore, it was important to determine whether this class of chemicals can also affect cell growth and differentiation in human cells. The present studies were undertaken to examine the response of human HL-60 promyelocytic leukemia cells (5) to a series of different tumor-promoting agents. These human cells were used because they display distinct biological and morphological commitments towards myeloid differentiation and can be efficiently induced to differentiate to mature cells by dimethyl sulfoxide and related compounds (11). Our studies with the phorbol diesters indicated that these tumor-promoting agents can efficiently induce terminal differentiation in the HL-60 cells. Cell differentiation could be detected at concentrations as low as 6×10^{-11} M. The fact that these chemicals act at doses comparable to the level of hormones in the cells suggests that they may mimic or compete with hormone-like chemicals that are involved in the control of cell growth and differentiation.

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MATERIALS AND METHODS

Chemicals. Phorbol-12-myristate-13-acetate [PMA; (12-tetradecanoyl-phorbol-13-acetate)], phorbol-12,13-diacetate, phorbol-12,13-didecanoate, phorbol-12-monomyristate, phorbol-12-monoacetate, phorbol-13-monoacetate, phorbol-13,20-diacetate, phorbol-12,13,20-triacetate, and phorbol-20-oxo-20-deoxy-12-myristate-13-acetate were obtained from Peter Borchert (University of Minnesota, Minneapolis). Phorbol-12,13-dibutyrate was provided by Thomas E. Slaga (Oak Ridge National Laboratory, Oak Ridge, TN). All the phorbol esters were dissolved in dimethyl sulfoxide, and the final concentration of this solvent in culture medium was 0.1%. The solvent at this concentration has no detectable effect on cell growth, phagocytosis, or morphological differentiation.

Cells and Culture Conditions. The human promyelocytic HL-60 leukemia cells were provided by Robert C. Gallo (National Cancer Institute, Bethesda, MD). The cells were cultured in tissue culture or bacterial petri dishes (no. 1007, Falcon) in RPMI-1640 medium plus 20% fetal calf serum supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) (GIBCO). Cell growth was determined from a count of the cell number after trypan blue-stained cells were excluded. For the experiments, 5×10^5 cells in their logarithmic growth phase were seeded in 5 ml of medium in 60-mm petri dishes and treated with the different phorbol esters 1 day after cells were seeded.

Evaluation of Cell Differentiation. The HL-60 cells grow in suspension as myeloblasts and promyelocytes. For morphological assessment of cell differentiation, control and treated cells were removed from the petri dishes and centrifuged into 1 ml of medium; aliquots of about 0.1 ml of the cell suspension were spread on a glass slide, dried in air, and stained with Wright/Giemsa. Differential cell counting was performed under a light microscope on 200–400 stained cells from at least two independent glass slides for each experimental point. Phagocytosis was determined after Wright/Giesma staining of control and treated cells that had been previously incubated in the 60-mm petri dishes for 30 min with 4×10^6 per ml of XY 664 auxotropic diploid *Saccharomyces cerevisiae*. The yeasts were provided by Jeffrey Lemontt (Oak Ridge National Laboratory, Oak Ridge, TN).

Abbreviation: PMA, phorbol-12-myristate-13-acetate (12-tetradecanoyl-phorbol-13-acetate).

RESULTS

Induction of Cell Differentiation by the Tumor Promoter PMA. To determine the effect of PMA on cell growth and differentiation, we incubated human HL-60 leukemia cells with different doses of the promoter in 60-mm tissue culture petri dishes. At 12 hr or more of treatment with doses of 10^{-10} M PMA or higher, the cells, which normally grow in suspension, attached to the surface of the tissue culture petri dishes, spread their cytoplasm, and formed pseudopodia-like structures. Attempts to remove the attached cells for cell counting by trypsinization or forceful pipetting with culture medium failed. To overcome this attachment problem, we performed experiments with bacterial plastic petri dishes, which have a lower surface tension. In these dishes there was a reduction in cell attachment and cytoplasmic spreading, and cells could be removed by pipetting with culture medium. All following experiments were therefore performed in the bacterial plastic petri dishes.

Untreated HL-60 cells contained mainly promyelocytes (70–80%), which are characterized by large nuclei (Fig. 1) (11). Treatment with PMA resulted in a cell progression towards more mature stages. The percent of myelocytes, metamyelocytes, and banded or segmented neutrophils (Fig. 1) increased with both PMA dose and time after treatment (Fig. 2 *A* and *B*). Induction of morphological differentiation could be determined after 1 day of treatment with a dose of 4×10^{-10} M or higher of PMA (Fig. 2*B*) and after 2 days with a dose as low as 6×10^{-11} M (Fig. 3). Treatment for 2 days with 8×10^{-10} M PMA resulted in a cell population that had about 90% of the cells in different stages of myeloid cell maturation (Fig. 2*B*). Induction of myeloid maturation by PMA in these cells was associated with an inhibition of cell growth (Fig. 2*A*). Treatment for more than 2 days with 4×10^{-10} M or more of this promoter resulted in cell degeneration. In view of this, all further experiments with tumor promoters were limited to 2 days of treatment.

In addition to the morphological analysis, HL-60 cells were tested for their phagocytic ability, which represents a physiological marker for myeloid differentiation (Fig. 2*C*). The results indicate that the percent of cells with the ability to phagocytize yeast cells was proportional to the dose of PMA. However, the percent of cells with phagocytic ability was less than half the percent that matured into myelocytes or more advanced stages (Fig. 2*B* and *C*). These results suggest that only a fraction of the mature cells, presumably the more advanced ones, gained the

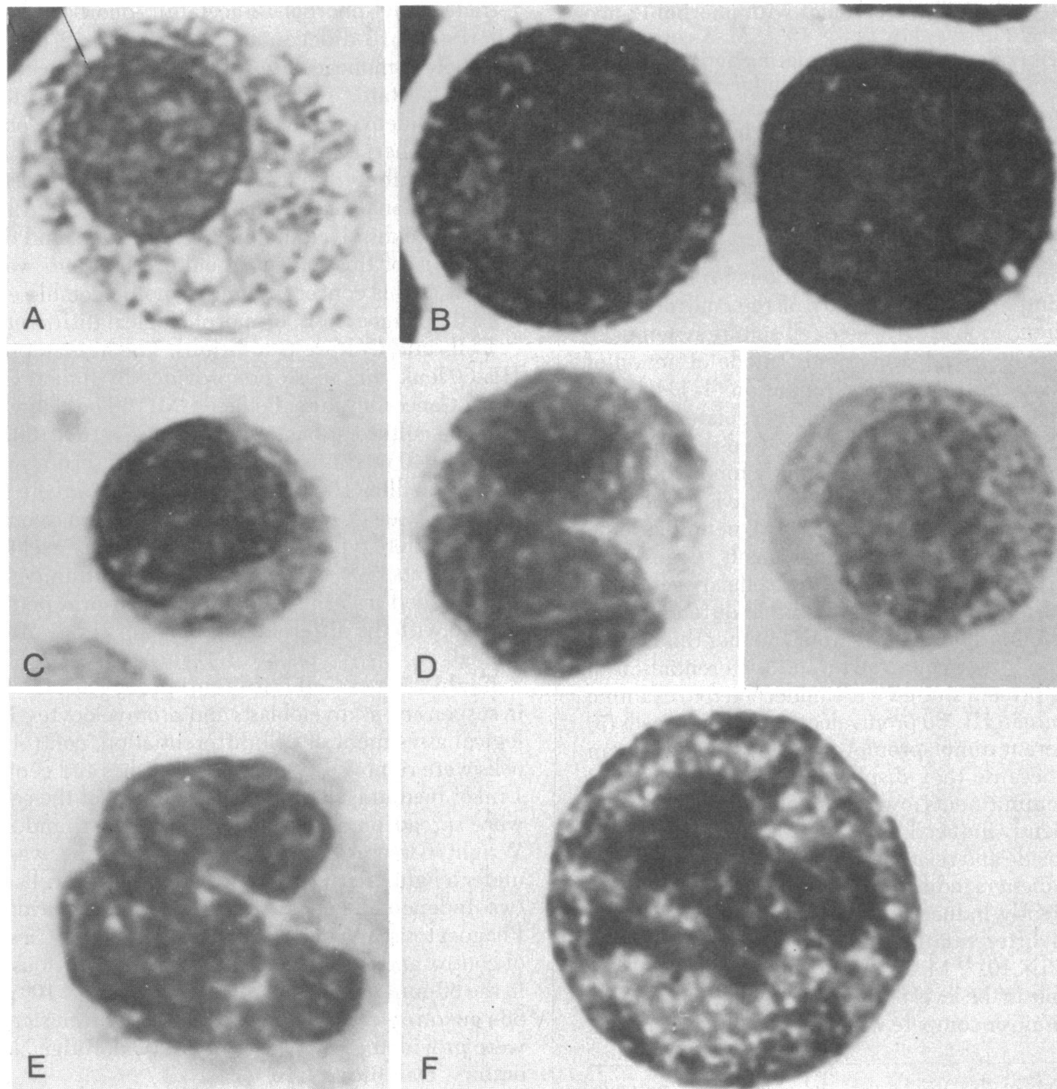

FIG. 1. Morphological myeloid differentiation in HL-60 cells. (*A*) Myeloblast, (*B*) promyelocytes, (*C*) myelocyte, (*D*) metamyelocytes, (*E*) banded neutrophil, and (*F*) segmented neutrophil. (×600.)

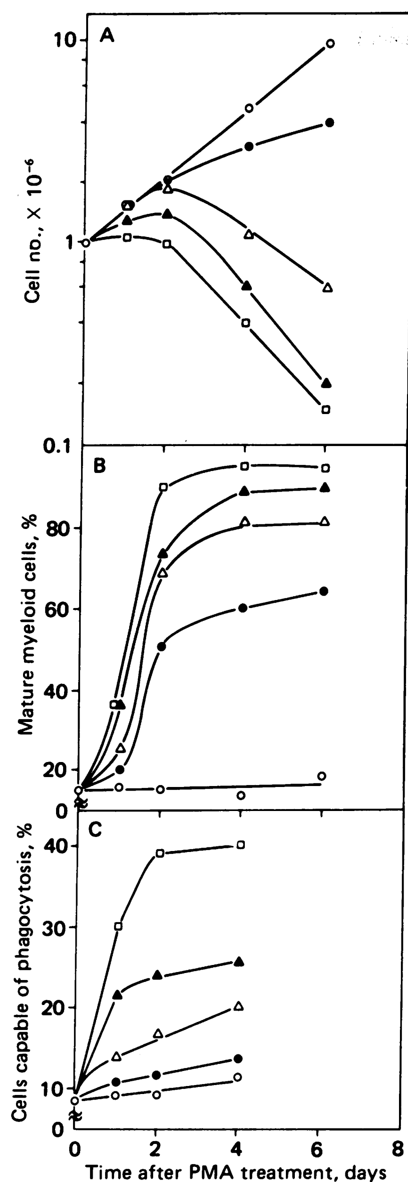

FIG. 2. Cell growth (*A*), differentiation (*B*), and phagocytosis (*C*) in HL-60 cells at different times after treatment with various concentrations of the tumor promoter PMA. The cultures were treated 1 day after 5×10^5 cells were seeded in 5 ml of growth medium in 60-mm petri dishes. Cells were counted to determine cell growth and stained with Wright/Giemsa stain to determine the percent of mature myeloid and phagocytizing cells. The mature myeloid cells were composed mainly from myelocytes and metamyelocytes. A small fraction of banded and segmented neutrophils was also observed. Phagocytosis was determined after the HL-60 cells were incubated for 30 min with 4×10^6 per ml of the diploid *S. cervisiae* stain XY 664. ○, Control. PMA: ●, 10^{-10} M △, 2×10^{-10} M; ▲, 4×10^{-10} M; □, 8×10^{-10} M.

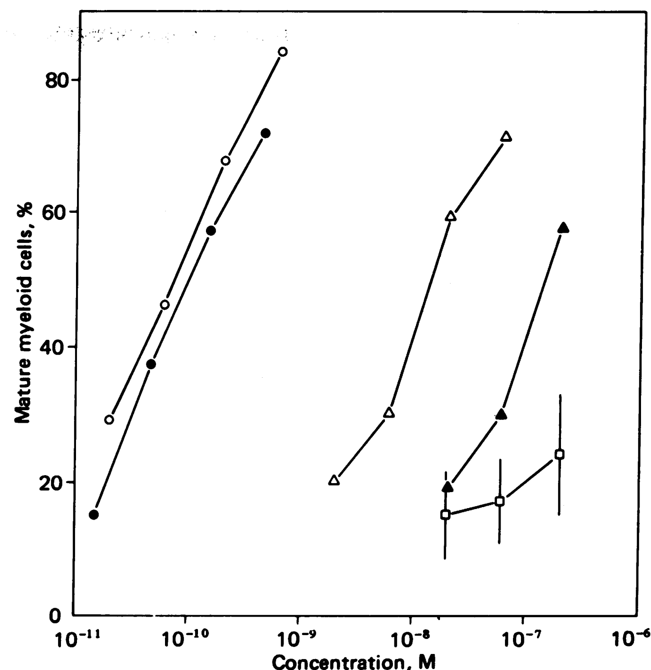

FIG. 3. Differentiation in the HL-60 cells 2 days after treatment with various concentrations of the phorbol esters. The percent of mature myeloid cells was determined after cells were stained with Wright/Giemsa. The control cultures contained about 15% mature myeloid cells. ○, PMA; ●, phorbol-12,13-didecanoate; △, phorbol-12,13-dibutyrate; ▲, phorbol-12,13-diacetate; □, phorbol-12-monomyristate, phorbol-12-monoacetate, phorbol-13-monoacetate, phorbol-13,20-diacetate, phorbol-12,13,20-triacetate, and phorbol-20-oxo-20-deoxy-12-myristate-13-acetate.

phagocytic ability. Our experiments on the induction of morphological myeloid cell maturation, phagocytosis, and inhibition of growth by PMA suggest that this tumor promoter induces in the promyelocytic leukemic cells terminal differentiation which resembles myeloid differentiation observed in normal hemopoietic cells.

Induction of Myeloid Cell Differentiation by Different Tumor Promoters. Induction of terminal differentiation in the HL-60 leukemia cells was also tested with nine other phorbol esters, some of which exhibit different degrees of tumor promotion in mouse skin (12, 13). The results indicate that three of the nine tested phorbol derivatives induced myeloid cell differentiation (Fig. 3 and Table 1) and inhibited cell growth. However, only phorbol-12,13-didecanoate was active at doses comparable to that of PMA. Phorbol-12,13-dibutyrate and phorbol-12,13-diacetate induced terminal differentiation at doses that were 2–3 orders of magnitude higher than those used for PMA. All other compounds were either negative or exhibited poor inducing activity (Fig. 3). These results indicate that the ability of these phorbol esters to induce terminal differentiation in the HL-60 cells is correlated with their tumor-promoting activity in mouse skin.

DISCUSSION

The present studies have shown that human HL-60 leukemia cells can be induced to differentiate into mature myeloid cells by a series of tumor-promoting agents. Differentiation was determined by cell morphology and by a physiological marker, phagocytosis. In addition to the changes noted above, the treated cells exhibited cell-surface changes characterized by attachment to the surface of the plastic petri dishes and cytoplasmic spread. These results suggest that alteration in cell-surface properties, which was also observed in other cells after treatment with tumor-promoting agents (19–21), may be the initial trigger that alters the growth properties of the cells, which in turn switch on the cellular program that results in the differentiated state. More specifically, this triggering may result from alterations caused by the tumor-promoting agents in the properties of receptor molecules that bind hormone-type growth factors present in the serum used for the cell culture or

Table 1. Myeloid differentiation in HL-60 cells after treatment with various phorbol diesters

Inducer	Tumor-promoting activity in mouse skin	Conc., M × 10^{10}	Myeloid cell type, %					
			Myeloblasts	Pro-myelocytes	Myelocytes	Meta-myelocytes	Banded neutrophils	Segmented neutrophils
None	−		10	74	12	4	0	0
PMA	++++	2	0	30	35	20	9	6
PDD	+++	1.5	0	43	31	18	5	3
PDB	+	200	2	40	37	16	3	2
PDA	−	2000	3	40	36	15	5	1

Differential myeloid cell counts were determined on cells stained with Wright/Giemsa 2 days after they were treated with the different phorbol diesters. PDD, phorbol-12,13-didecanoate; PDB, phorbol-12,13-dibutyrate; PDA, phorbol-12,13-diacetate.

by their competitive binding to such receptors. The latter possibility seems to be of interest in view of the recent experiments reported by Lee and Weinstein (22) in which tumor-promoting agents inhibited binding of epidermal growth factor to cellular receptors of HeLa and xeroderma pigmentosum cells. There is, however, also the possibility that in the process of malignant cell transformation (10, 23–27) receptors to inducers of differentiation in the leukemic cells either are not available or are completely lost. Examples for such cases are Friend mouse erythroleukemia cells and some mouse myeloid leukemia cells, which do not respond to erythropoietin (28), and macrophage and granulocyte inducer protein (10), which are presumably inducers of normal erythroid and myeloid differentiation, respectively. Tumor promoters may thus either overcome the step of interaction of the inducers for differentiation with the appropriate receptors or alter the cell surface in such a way as to expose such receptors for an interaction with the inducers that may be present in the serum used for the cell culture.

The present experiments indicate that the tumor-promoting phorbol diesters induce terminal differentiation in the human HL-60 cells; in other studies in which avian and murine cells were used, usually they did not induce, but in fact inhibited, differentiation (14–18, 29). These results imply that either myeloid leukemia cells respond differently from other cell types or that human cells, including the HL-60 promyelocytic leukemia cells, respond differently from mouse and chicken cells to the biological effect of phorbol diesters. This latter possibility is not surprising in view of the experiments in which it was shown that rabbits and guinea pigs did not respond to the tumor-promoting effect of croton oil (30), of which PMA is the major active component (12). In view of this possibility, it is interesting to note the relatively low doses of PMA (6×10^{-11}–8×10^{-10} M) that are required to inhibit cell growth and induce cell differentiation in the HL-60 cells. Preliminary studies indicated that terminal differentiation could be obtained not only in HL-60 cells, but also in the human K562-4 myeloid leukemia cells (31) after treatment with the above doses of PMA (unpublished results). Normal human fibroblasts are susceptible to the growth-inhibitory effect of PMA at doses of 100- to 1000-fold higher than those used in these studies (32). Based on these observations, it is possible to speculate that HL-60 and K562-4 leukemia cells, and perhaps other human tumor cells, may be more susceptible than normal cells to the growth inhibition of PMA, thus offering an approach to control myeloid leukemia and perhaps other types of tumors. However, studies on the susceptibility of a variety of different normal and malignant human tissues to the growth-inhibitory effect of these types of compounds have to be performed before such an approach can be considered.

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