Identification of a population of bipotent stem cells in the HL60 human promyelocytic leukemia cell line

(phorbol esters/commitment/differentiation/dimethyl formamide)

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ABSTRACT HL60, the human promyelocytic leukemia cell line, consists of cells that resemble promyelocytes but are transformed into more mature myeloid forms (polymorphonuclear leukocytes) that express most of the functional characteristics of terminally differentiated myeloid forms when incubated in the presence of dimethyl formamide (HCONMe₂). When HL60 cells are exposed to the phorbol diester 12-O-tetradecanoylphorbol 13-acetate [phorbol 12-myristate 13-acetate (PMA)], they shift from suspension to adherent on the surface of the tissue culture vessel, assume the appearance of macrophages, and acquire macrophage-associated surface markers, the enzyme-nonspecific esterase, the isozyme acid phosphatase, and the myeloid-specific esterase. Although data from other laboratories suggest that HL60 consists of stem cells that are bipotent with respect to myeloid or macrophage differentiation, unequivocal proof of bipotency or evidence that ruled out the presence of two different types of stem cells, each committed to different lines of hematopoietic differentiation, has been lacking. To resolve these two alternatives, we have developed a cloned population of HL60 cells and studied the properties of these cells when exposed to inducers of myeloid (HCONMe₂) or macrophage (PMA) differentiation. After 120 hr of incubation with HCONMe₂, 95% of the cells acquire myeloid markers and lack specific macrophage markers, whereas the reverse is true in the presence of PMA, clearly establishing that HL60 is able to commit itself to the development of two different programs of hematopoietic differentiation. Moreover, the commitment to macrophage differentiation is irreversible after 6 hr of incubation in PMA whereas development of the myeloid program requires continuous exposure of the cells to HCONMe₂. Commitment of HL60 to macrophage differentiation by PMA is not affected by subsequent addition of HCONMe₂, whereas HCONMe₂-induced myeloid differentiation is overridden by exposure of the cells to PMA. These data suggest that the HL60 cell may provide a model system for studying the process that generates several classes of precursor cells, each committed to different lines of hematopoietic differentiation, as well as the process that mediates the maturation of these committed precursor cells to their terminally differentiated state.

The mechanism by which hematopoietic pluripotent stem cells become committed to the development of independent lineages of differentiated hematopoietic cells is unknown. Two important models for hematopoietic differentiation have been proposed: the instructional model and the stochastic model. In the former, an uncommitted hematopoietic stem cell is multipotent but becomes committed to a specific line of hematopoietic differentiation (e.g., erythroid, myeloid, lymphoid, monocytoid) when exposed to some extracellular environmental stimulus (such as hormones or cell-to-cell contact). This model assumes the existence of single cells committed to more than one lineage

of hematopoietic differentiation. In the second model, a stochastic event of unspecified mechanism determines, during some portion of the cell cycle, that one or more of the daughter cells of a mitotic division become committed to a single line of hematopoietic differentiation. In this model, the population will be dominated by cells exhibiting commitment to a single pathway of hematopoietic differentiation.

It was thus of interest that HL60, a continuously proliferating suspension cell line isolated by Collins *et al.* (1), was shown to develop the cellular functions of mature myeloid cells when incubated in dimethyl formamide (HCONMe₂) (2), whereas it developed the cellular functions, surface markers, morphology, and adherent growth properties of macrophages when incubated in the presence of the phorbol diester 12-O-tetradecanoylphorbol 13-acetate [phorbol 12-myristate 13-acetate (PMA)] (3-6). These initial studies did not specify whether the HL60 population used was a mixture of cells, one committed to myeloid and the other committed to monocytoid differentiation, or a homogeneous population of bipotent cells, capable in the presence of the appropriate agent of developing into either macrophages or polymorphonuclear leukocytes.

To resolve this question, we isolated a cloned population of HL60 cells from the original inoculum isolated by Collins *et al.* (1), which, after cloning from single cells, resembled human promyelocytes and grew in suspension culture. We then studied the behavior of this cloned homogeneous population of cells in the presence of PMA and HCONMe₂ to determine whether this clone could develop the cellular function of macrophages or myeloid cells, respectively. Our studies have shown that, in the presence of PMA, >90% of the cloned cells resemble macrophages and possess the surface markers and cellular functions characteristic of mature macrophages while <20% of the PMA-incubated cells develop the myeloid markers of nitroblue tetrazolium (NBT) reduction and specific esterase activity. In contrast, incubation of these cloned HL60 cells in HCONMe₂ showed that >90% of the cells have these myeloid markers but lack the markers of macrophage differentiation. These studies thus demonstrate that the HL60 cell line contains cells capable of differentiation along two separate lines of hematopoietic differentiation and that these cells exhibit differences with respect to the presence of commitment to each line of differentiation.

MATERIAL AND METHODS

The Epstein-Barr virus-transformed human lymphoblast cell line 2292 was obtained from the Institute of Medical Research

Abbreviations: PMA, phorbol 12-myristate 13-acetate (12-O-tetradecanoylphorbol 13-acetate); HCONMe₂, dimethylformamide; NBT, nitroblue tetrazolium.

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through the courtesy of Jay Tischfield and maintained in RPMI 1640 (National Institutes of Health Media Unit)/10% fetal calf serum (GIBCO). All cultures were free of mycoplasma. Induction of cells was performed by inoculating cells in their respective media at 5×10^4 cells per ml and, after two generations (60 hr), the inducer was added. HCONMe₂ (Sigma) was added to a final concentration of 120 mM. This concentration was chosen because our experiments had shown that full induction of markers of myeloid differentiation does not occur at concentrations less than 120 mM and that significant increases in cell death occur at concentrations greater than 120 mM. PMA (Sigma) was added to a final concentration of 64 mM.

Histochemistry. Naphthol AS-D chloroacetate (specific) esterase was assayed as described by Yam *et al.* (7). α -Naphthol AS-D acetate (nonspecific) esterase was assayed as described by Li *et al.* (8). Myeloperoxidase was assayed by the procedure of Kaplow (9). The ability of cells to reduce NBT was assayed according to the procedures of Newberger *et al.* (10).

Cloning of Cells. One hundred HL60 cells in log phase of growth were plated in each of several 60-mm Petri dishes in 5 ml of medium and incubated for 12 hr. The dishes were then screened with an inverted phase microscope to identify single cells in areas devoid of additional cells. Cloning cylinders were placed over each single cell, and the contents covered by the cloning cylinder was checked by microscopic examination to ascertain that only a single cell was present in the area confined. The contents of cylinders shown to contain only a single cell were then transferred to the wells of a flat-bottomed multiwell plate (each well contained 0.5 ml of medium). After the transfer, each well was checked to ensure that it contained only a single cell. Populations of cells were grown 10–14 days after the cloning and then transferred to mass culture in T35 flasks.

RESULTS

Behavior of HL60 Clone in the Presence of PMA and HCONMe₂. We first exposed the HL60 clone to inducers of myeloid (HCONMe₂) and macrophage (PMA) differentiation. As shown in Table 1, >90% of the HCONMe₂-induced cells had expressed markers of myeloid differentiation [NBT reduction, naphthol AS-D chloroacetate (specific) esterase activity] but <2% of these cells were positive for markers of macrophage differentiation [alpha naphthol AS-D acetate (nonspecific) esterase activity, growth as adherent cells]. In the presence of PMA, essentially the entire population acquired the properties characteristic of macrophages, including nonspecific esterase

FIG. 1. Differentiation of HL60 cells after exposure to HCONMe₂ for various time periods. Cells were incubated in the presence of HCONMe₂ and harvested at various times. Differentiation as indicated by NBT reduction was determined in a portion of the cells immediately on harvesting (○) at the conclusion of the incubation. The remainder of the cells were washed free of HCONMe₂ and further incubated for a total incubation period of 120 hr and then assayed for NBT reduction (●).

activity and growth as adherent cells. Less than 20% of these cells showed characteristics of myeloid cells, such as specific esterase and NBT reduction. PMA-induced cells continue to express myeloperoxidase activity, which indicates that the loss of myeloid markers is not due to PMA-induced degranulation but is exerted at the level of expression of the active enzymes themselves. These results suggest that the HL60 clone studied is a bipotent cell with respect to differentiation into macrophages and mature myeloid cells.

Kinetics of HCONMe₂ or PMA Induction. The kinetics of appearance of the markers of myeloid and monocyte differentiation with HCONMe₂ and PMA are different. As shown in Fig. 1, 96 hr of continuous incubation of HCONMe₂ are required to develop the mature myeloid phenotype. Table 2 shows the kinetics of PMA-induced differentiation. In these experiments, cells were exposed to PMA for 2–12 hr; then, they were washed and further incubated in the absence of PMA until 24 hr had elapsed from the beginning of the PMA incubation. After a 2-hr incubation with PMA, 26% of the cells became committed to the macrophage state, as shown by their growth as adherent cells and the acquisition of the macrophage marker nonspecific esterase. After a 10-hr period of PMA incubation, >50% of the cells in the population became committed to the development of macrophage cellular functions and surface markers. Thus, after an initial exposure to PMA, the cells become committed to macrophage development even when it is removed from the medium.

As shown in Table 2, only those cells that are adherent express the macrophage marker nonspecific esterase and lose the specific esterase myeloid marker. Thus, adherent growth, nonspecific esterase expression, and loss of expression of specific esterase occur in a coordinate fashion after PMA-induced commitment to macrophage differentiation.

Interaction of PMA and HCONMe₂. In view of the ability of HL60 to differentiate along two different pathways, it was of interest to determine whether the induction by one agent was influenced by the presence of the other. In these experiments, cells were incubated for 96 hr in HCONMe₂ and exposed to PMA during the incubation period for four different time periods: 0–24 hr, 24–48 hr, 48–72 hr, and 72–96 hr (Table 3). Up to 48 hr after the addition of HCONMe₂, the addition of PMA results in the appearance of macrophage markers: growth as adherent cells, induction of nonspecific esterase, and loss of specific esterase in >90% of the cells. However, after 72 hr of exposure to HCONMe₂, when the HL60 cells initially express

Table 1. HCONMe₂- and PMA-induced HL60 cells: Expression of myeloid and macrophage phenotypes

Induction agent (period)	Macrophage marker		Myeloid marker	
	Adherent	NSE activity	SE activity	NBT reduction
None	0	2 ± 1	99 ± 1	2 ± 2
HCONH ₂ (96 hr)	0	4 ± 2	99 ± 1	90 ± 12
PMA (24 hr)	95 ± 6	90 ± 10	30 ± 10	15 ± 10
PMA (48 hr)	95 ± 6	90 ± 6	4 ± 2	20 ± 10
PMA (72 hr)	95 ± 7	90 ± 4	4 ± 2	15 ± 8

At least 200 cells were assessed for nonspecific esterase (NSE) and specific esterase (SE) activities. All cell populations studied were composed of cells >90% of which were positive for myeloperoxidase. Results are expressed as percent of cells exhibiting the marker and represent mean ± SEM of four determinations.

Table 2. Kinetics of PMA-induced development of the macrophage phenotype in HL60

Duration of exposure, hr	Marker						NSE positive in B population	
	Adherent		NSE activity		SE activity			
	A	B	A	B	A	B	Adherent	Suspension
2	<1	26 ± 4	5 ± 3	26 ± 4	80 ± 20	70 ± 10	95 ± 3	1 ± 1
4	<1	36 ± 10	4 ± 4	33 ± 10	80 ± 20	65 ± 15	94 ± 4	8 ± 6
6	<1	40 ± 15	7 ± 4	40 ± 10	80 ± 22	70 ± 11	96 ± 4	10 ± 4
8	4 ± 2	42 ± 15	8 ± 7	45 ± 10	60 ± 15	52 ± 20	97 ± 3	12 ± 6
10	5 ± 4	54 ± 16	10 ± 8	50 ± 10	56 ± 18	50 ± 16	96 ± 4	12 ± 6
12	8 ± 6	60 ± 18	10 ± 10	60 ± 10	50 ± 15	40 ± 13	96 ± 4	12 ± 8
24	94 ± 5	95 ± 3	96 ± 5	96 ± 4	35 ± 15	34 ± 20	92 ± 6	ND

(A) Cells were assayed for adherence, nonspecific esterase activity (NSE), and specific esterase activity (SE) immediately after removal of PMA. (B) Cells were assayed for adherence, NSE activity, and SE activity after removal of PMA and further incubation in its absence to a total incubation time of 24 hr. Results are expressed as percent of cells exhibiting the marker and represent mean ± SEM of four determinations.

orientation, chemotaxis, and the ability to reduce NBT (5), the addition of PMA continues to result in marked adherence in most cells, although induction of nonspecific esterase activity occurs in a smaller, but still significant, percentage of cells. As opposed to the dominant effect of PMA on HCONMe₂ induction, the addition of HCONMe₂ to PMA-induced cells does not affect PMA induction of adherence, nonspecific esterase activity, and loss of specific esterase activity (Table 4).

Stimulation of [³H]Leucine Incorporation. As we have reported (6), induction of HL60 cells with HCONMe₂ results in a dramatic stimulation in the rate of leucine incorporation into protein. When the rate of incorporation of [³H]leucine into trichloroacetic acid-precipitable protein was determined in our cloned population after the addition of PMA, a stimulation in the rate of [³H]leucine incorporation was also noted (Fig. 2). To determine whether the PMA effect was specific for macrophage differentiation and not due to a nonspecific effect of PMA on cells, we exposed two additional hematopoietic cell lines of differentiated states to [³H]leucine after PMA exposure. The addition of PMA to cultures of mouse erythroleukemia cells did not result in either a decrease in growth rate, a change in the morphological appearance of the cells, the induction of benzidine-positive cells, or stimulation in the rate of [³H]leucine incorporation into protein. However, when PMA was added to cultures of a lymphoblast cell line with surface marker characteristics of B cells (2292), we observed marked clumping of the cells without surface adherence, an increase in the expression of the Epstein-Barr virus antigen (data not shown), and stimulation of the rate of [³H]leucine incorporation into 2292 cells. Thus, stimulation of incorporation of [³H]leucine is dependent on the differentiated state of the cell tested (e.g., erythroid, lymphoid, or macrophage) and is not a nonspecific membrane effect of PMA on all cells.

Table 3. Effect on HL60 of sequential exposure to inducing agents: Addition of PMA after various periods of HCONMe₂ induction

Period of PMA exposure, hr	Marker		
	Adherence	NSE activity	SE activity
0-24	90 ± 5	96 ± 4	5 ± 5
24-48	95 ± 5	96 ± 3	3 ± 4
48-72	90 ± 8	70 ± 20	2 ± 3
72-96	92 ± 4	20 ± 10	40 ± 18

Results are expressed as percent of cells exhibiting the marker and represent mean ± SEM of four determinations. NSE, nonspecific esterase; SE, specific esterase.

DISCUSSION

By using a cloned population of HL60 cells and inducers of myeloid and macrophage differentiation, we have shown that the HL60 cell line contains cells that are bipotent with respect to hematopoietic differentiation. The cells morphologically resemble promyelocytes in the uninduced state and are capable of developing into two different types of differentiated hematopoietic cells. Our data have further shown that development of the macrophage phenotype in the presence of PMA involved a commitment step beyond which the continued presence of PMA is no longer required. Finally, we have found that the PMA and HCONMe₂ induction of macrophage and myeloid phenotypes in HL60 differs with respect to the presence of such a commitment step or at least in the kinetics of its development.

Studies involving the *in vitro* growth of normal bone marrow cells have previously suggested that the myeloid and monocytic lineages share a common ancestor in hematopoietic differentiation (11, 12). Ichikawa (13) has isolated numerous normal and leukemic mouse cell lines, which have been shown by Fibach and Sachs (14) to generate colonies composed of mixtures of myeloid and monocytic cells in the presence of serum growth factors. Our data suggest that the HL60 cell line is derived from the myeloid-macrophage common ancestral stem cell and that the development of mature macrophages and polymorphonuclear leukocytes from the common stem cell proceeds in the HL60 cell in a manner consistent with an instructional model of hematopoietic differentiation.

Our studies have shown that the HL60 cell becomes committed to the development of the macrophage program of differentiation after 6 hr of exposure to PMA and that the development of mature macrophages continues even if the agent is removed from the cultures at this time. The absence of commitment to macrophage differentiation in a majority of cells with

Table 4. Effect on HL60 of sequential exposure to inducing agents: Addition of HCONMe₂ after various periods of PMA induction

Period of HCONMe ₂ exposure, hr	Marker		
	Adherence	NSE activity	SE activity
None	98 ± 3	96 ± 3	22 ± 13
0-24	98 ± 4	96 ± 2	15 ± 10
24-48	96 ± 3	95 ± 4	6 ± 2
48-72	92 ± 6	98 ± 2	4 ± 2
72-96	97 ± 2	97 ± 2	3 ± 3

Results are expressed as percent of cells exhibiting the marker and represent mean ± SEM of four determinations.

FIG. 2. [^3H]leucine incorporation into HL60 (C), mouse erythroleukemia cells (A), and a lymphoblast line (B) after exposure to PMA for 24, 48, and 72 hr. Cells (1×10^6) suspended in 1 ml of Gey's balanced salt solution were preincubated at 37°C , and 0.5 Ci ($1 \text{ Ci} = 3.7 \times 10^{10}$ becquerels) of [^3H]leucine was added to the suspension. The amount of radioactive precursor incorporated ($\square$) was determined by addition of trichloroacetic acid, followed by washing the precipitates with trichloroacetic acid and dissolving them 0.5% NaDodSO_4 for counting. The number of hours during which each cell line was exposed to PMA is indicated (24, 48, or 72 hr). $\blacksquare$, Control (absence of PMA).

PMA exposure times of less than 6 hr shows that the commitment seen for PMA exposure times of more than 6 hr is not just due to irreversible binding or incorporation of PMA by the cells; rather, commitment is generated by a change in the cell itself.

In contrast, HCONMe₂-induced development of mature myeloid cells in HL60 requires the continuous presence of the inductive agent, unlike the induction of myeloid differentiation by retinoic acid (15), which has been shown by others to be irreversible. Thus, we are unable to define a commitment step to myeloid differentiation in HL60, perhaps because the HL60 cell has already started to proceed along this pathway, which suggests that HCONMe₂ acts merely to increase the percentage of cells in culture that mature along this pathway.

It is interesting, therefore, that the addition of HCONMe₂ to HL60 cells is without effect once the cells have been committed to macrophage development in the presence of PMA.

Conversely, the addition of PMA to cultures of HL60 that are undergoing myeloid maturation reduces the percentage of cells exhibiting a myeloid phenotype as it increases that of cells exhibiting a macrophage phenotype. The dominance of the effect of PMA over that of other agents affecting the differentiation and maturation of HL60 cells has recently been observed in a study of PMA and retinoic acid, an inducer of myeloid differentiation (15), on an uncloned population of HL60 cells (3). It is not possible to conclude whether this dominance of PMA is due to a difference in the mechanism through which PMA and HCONMe₂ affect the HL60 cells, the presence of high- and low-affinity receptors on HL60 for PMA and HCONMe₂, respectively, or the differentiated state of the HL60 cell that endows it with a proclivity to develop into macrophages and myeloid cells. These studies of the interaction of PMA and HCONMe₂ during the induction of differentiated phenotypes of HL60 also suggest that the commitment to a program of differentiation may be separable in these experiments from its expression (i.e., early appearance of a HCONMe₂-induced phenotype may be reversed by exposure to PMA). Finally, our studies of PMA-induced stimulation of [^3H]leucine incorporation in cells of different states of differentiation (erythroid, lymphoid, and macrophage) suggest that the response of cells to PMA (e.g., dominance of PMA over HCONMe₂) depends on the differentiated state of the cells that exists before their exposure to the inducer and does not arise from a general nonspecific effect of the inducer that is unrelated to the differentiated state of the cell. The ability to program and reprogram the differentiated state of these cloned HL60 cells suggests that further studies with this system may lead to an understanding of the molecular basis of commitment and maturation in differentiating hematopoietic cells.

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