Polyamine Requirements for Induction of HL-60 Promyelocyte Differentiation by Leukocyte-conditioned Medium and Phorbol Ester

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ABSTRACT

The polyamines putrescine, spermidine, and spermine have been implicated in the regulation of both proliferation and differentiation. Spermidine is required for DNA replication, and the intracellular depletion of this polyamine can be used to distinguish cellular events related to proliferation. We have demonstrated previously that depletion of intracellular spermidine results in cytostasis of human HL-60 promyelocytes. The inhibition of HL-60 proliferation is associated with inhibition of differentiation induced by dimethyl sulfoxide, hexamethylene bisacetamide, butyric acid, and retinoic acid. The present studies extend these findings by monitoring the effects of spermidine depletion and inhibition of DNA replication on induction of HL-60 differentiation by leukocyte-conditioned medium and phorbol ester. The results demonstrate that both inducers enhance intracellular polyamine levels. However, depletion of intracellular spermidine inhibits induction of HL-60 differentiation by leukocyte-conditioned medium, but not by phorbol ester. Further, the addition of exogenous spermidine abrogates the inhibition of proliferation, and leukocyte-conditioned medium induced HL-60 differentiation, confirming the requirement of this polyamine for the expression of a differentiated phenotype. The present results and our previous findings suggest that spermidine is required for induction of HL-60 differentiation by a variety of agents, including leukocyte-conditioned medium, but that phorbol ester induces HL-60 differentiation in the absence of polyamine biosynthesis and DNA replication.

INTRODUCTION

The polyamines putrescine, spermidine, and spermine have been implicated in the regulation of cellular proliferation (10, 25) and differentiation (2, 6, 7, 26, 29). The activity of the rate-limiting enzyme in the polyamine synthetic pathway, ODCase, is increased prior to cellular proliferation, and inhibition of this enzyme by DFMO is associated with an arrest of cell growth (14-16). Spermidine is required for cell proliferation and reverses cytostasis induced by DFMO (19). Thus, the reversible depletion of intracellular spermidine by DFMO inhibits DNA replication, and this approach has been used to demonstrate the requirement of spermidine and proliferation for the induction of MEL cell differentiation by DMSO, HMBA, and BA (23).

The human HL-60 promyelocytic cell line has also been studied as a model of myeloid differentiation (1). Previous studies have indicated that the induction of HL-60 differentiation by DMSO, RA, and BA is not inhibited by a block in cellular proliferation induced by hydroxyurea and 1-β-D-arabinofuranosylcytosine (4). It has also been demonstrated that phorbol ester-induced macrophage differentiation of HL-60 cells can occur in the absence of DNA synthesis (20, 27). In contrast, we have inhibited HL-60 proliferation by exposing cells to DFMO and have demonstrated that depleting intracellular spermidine blocks induction of HL-60 differentiation by DMSO, HMBA, BA, and RA (24). We have also demonstrated a highly significant relationship between inhibition of both proliferation and induction of HL-60 differentiation by these agents (24), thus suggesting that replication is required for expression of a differentiated HL-60 phenotype. Similarly, induction of HL-60 cells along the monocyte-macrophage lineage by lymphocyte-conditioned medium has been shown by DNA histogram analysis to require S-phase synthesis (31).

The relationship between proliferation and differentiation of HL-60 cells has therefore not been clearly established. In order to address this issue, we have examined HL-60 differentiation induced by either LCM or phorbol ester following complete inhibition of DNA synthesis and cellular proliferation by the depletion of intracellular spermidine. The results demonstrate that spermidine is required for induction of HL-60 differentiation by LCM but that phorbol ester induces HL-60 differentiation in the absence of this polyamine and DNA replication.

MATERIALS AND METHODS

Cell Culture. HL-60 cells were grown in RPMI-1640 medium (Grand Island Biological Co., Grand Island, NY) containing 4 mM L-glutamine (Grand Island Biological Co.), 1% penicillin-streptomycin (Grand Island Biological Co.), 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate in 20% heat-inactivated fetal bovine serum (Grand Island Biological Co.) at a density ranging from 1 to 2 x 10^5/ml in a 5% CO₂-humidified atmosphere at 37°C. Viable cells were determined by trypan blue exclusion. DFMO (RF-71, 72A; Merrell Dow Pharmaceuticals, Cincinnati, OH) was added at a final concentration of 5 x 10⁻³ M with each passage 60, 36, and 12 hr prior to the addition of inducer. Phytomethylglutamin-stimulated LCM was prepared as described previously (28) and diluted to 20% in the HL-60 culture medium. TPA (Sigma Chemical Co., St. Louis, MO) was dissolved in acetone at 3.2 x 10⁻⁶ M and then further diluted to 3.2 x 10⁻⁸ M in the culture medium.

Histochemical Staining. Cytocentrifuge smears of culture cells were examined for the expression of a-naphthyl acetate esterase (nonspecific esterase) and NBT reduction (30). The percentage of positive cells was determined by counting 200 cells in duplicate.

Monoclonal Antibodies. The monoclonal antibody Mo1 was prepared...
and used as a measure of HL-60 differentiation as described previously (28). Mo1 antigen is expressed by monocytes, granulocytes, null cells in the peripheral blood, and immature myeloid cells in the bone marrow. Monoclonal antibody MY4 reacts with the cell surface of monocytes (8). Antigen expression was detected by an indirect immunofluorescence assay using an Ortho Cytofluorogar FC200/4800A (Ortho Instruments, Westwood, MA). Background fluorescence was determined using a nonreactive IgG antibody. Ten thousand cells were analyzed per sample.

Measurement of Intracellular Polyamines. HL-60 cells (4 x 10⁶) were harvested and washed twice with Dulbecco 'A' phosphate-buffered saline (Oxoid Limited, England). Polyamines were extracted in 4% sulfosalicylic acid and separated on a Beckman 121 MB amino acid analyzer (8). The levels were quantitated using 3,3'-diaminodipropylamine as an internal standard. Putrescine, 3,3'-diaminodipropylamine, spermidine, and spermine are detectable at 22.5, 30.7, 43.7, and 44.8 min, respectively. The results (pmol/10⁵ cells) are expressed as the mean ± S.D. for 2 separate determinations, each performed in duplicate.

RESULTS

We have monitored the effects of LCM and TPA on intracellular polyamine levels in HL-60 cells (Chart 1). Putrescine increased approximately 2.5-fold at 6 hr after adding LCM, while TPA resulted in less pronounced increases in the level of this polyamine (Chart 1A). Similarly, intracellular spermidine (Chart 1B) increased following exposure to either LCM or TPA. In contrast, TPA had no detectable effect on spermine levels (Chart 1C), while increases in this polyamine were observed following exposure to LCM. These results suggest that exposure of HL-60 cells to either LCM or TPA is associated with increases in polyamine synthesis.

The effects of 5 mm DFMO on intracellular polyamine levels were also monitored in control and LCM- or TPA-treated HL-60 cells (Chart 2). The cells were treated with DFMO for 60 hr prior to the addition of inducer. Under these conditions, intracellular putrescine (Chart 2A) and spermidine (Chart 2B) were undetectable at the time of treatment with LCM or TPA. Further, these polyamines remained undetectable during the period monitored for induction of differentiation (LCM, 96 hr; TPA, 48 hr). Treatment with DFMO also resulted in decreased intracellular spermine levels. However, spermine remained detectable and was similar in the LCM- or TPA-treated cells (Chart 2C).

The effects of DFMO, LCM, and TPA on HL-60 proliferation are illustrated in Chart 3. Proliferation of cells treated with LCM was similar to that of untreated cells over a 72-hr period. The DFMO-treated cells achieved cytostasis at the time of LCM addition, and there was no subsequent proliferation. In contrast, TPA inhibited HL-60 proliferation (Chart 3B), and similar results were obtained with HL-60 cells treated with both DFMO and TPA. Further, the results obtained with DFMO-treated cells were similar to those obtained with DFMO-LCM- or DFMO-TPA-treated cells.

The induction of HL-60 differentiation by LCM and TPA was monitored using monoclonal antibodies anti-MY4 and anti-Mo1. Chart 4 illustrates the pattern of reactivity obtained after 96 hr of exposure to LCM. The cell surface expression of MY4 was
Polyamine Requirements for HL-60 Differentiation

The HL-60 cells were also monitored for NSE and NBT staining. DFMO alone had no significant effect on histochemical staining. The exposure of HL-60 cells to LCM for 96 hr resulted in significant increases in both NSE and NBT staining, consistent with maturation along both the monocyte-macrophage and myeloid lineages (Table 1). The expression of each of these parameters was inhibited by DFMO treatment. Further, the inhibition of cell surface antigen expression and histochemical staining by DFMO was abrogated by the addition of exogenous spermidine, thus confirming the involvement of this polyamine in the expression of the differentiated phenotype.

Table 1 lists the results obtained following exposure of HL-60 cells to TPA for 48 hr. Staining for NBT reduction after TPA exposure was similar to control levels. The percentage of cells adhering to the tissue culture flask was also monitored as another parameter of monocyte-macrophage maturation. In these experiments, the expression of Mo1 antigen, NSE staining, and the percentage of adherent cells increased with TPA exposure. More importantly, DFMO treatment had no effect on the expression of these markers of monocyte-macrophage differentiation.

DISCUSSION

We have monitored previously the relationship of intracellular polyamine levels with proliferation and differentiation of MEL cells (23). The results demonstrated a significant relationship between intracellular spermidine levels and MEL proliferation. The results also demonstrated that spermidine was required for induction of MEL differentiation by DMSO, HMBA, and BA. A significant relationship between MEL proliferation and differentiation suggested that spermidine was involved primarily in proliferation and, thereby, differentiation. It was not possible to determine the requirements of spermidine and proliferation in the induction of HL-60 differentiation by DMSO, HMBA, and BA (24).

The arrest of HL-60 cellular proliferation by depletion of intracellular polyamines has been used in the present studies to determine the requirements of spermidine and proliferation in the induction of HL-60 differentiation by LCM and TPA. The results demonstrate that depletion of intracellular spermidine blocks the induction of a differentiated phenotype by LCM. Further, repletion of intracellular spermidine reverses cytostasis and results in the expression of cell surface and histochemical markers of both monocyte-macrophage and granulocyte differentiation. These findings suggest that induction of HL-60 differentiation by LCM requires spermidine and proliferation. Similarly, polyamines have been shown to play an essential role in the induction of both proliferation and granulocytic differentiation of normal human bone marrow progenitor cells by placenta-conditioned medium (29).

In contrast, depletion of intracellular polyamines had no effect on the induction of a differentiated phenotype by TPA, thus suggesting that induction of differentiation by this agent does not require spermidine and cell replication. This finding confirms previous observations that polyamines (13) and cell proliferation (20, 27) are not required for TPA induction along the monocyte-macrophage lineage. Thus, the induction of HL-60 differentiation by TPA probably differs considerably from the mechanisms of the other inducers that require spermidine and proliferation. It is possible that TPA activates a late step in the pathway of events leading to differentiation by bypassing early events involving DNA replication. The recent demonstration that TPA activates a cell membrane protein kinase in certain cells (5) is of interest in this regard, and the possible role of such protein kinases in myeloid differentiation requires further investigation.

Finally, previous reports have supported the dissociation of HL-60 proliferation and differentiation (4, 11, 13). Thus, the arrest of HL-60 proliferation by hydroxyurea and 1-β-D-arabinofuranose...
sylcytosine did not block induction of differentiation by DMSO, RA, and BA (4). Although increases in ODCase activity have been shown to be essential for proliferation and not differentiation (11, 13), polyamine levels were either not monitored (11) or only partially depleted upon addition of the inducer (13). In contrast, we have completely depleted intracellular polyamines to demonstrate the requirements of spermidine for both proliferation and induction of HL-60 differentiation with DMSO, HMBA, BA, RA, and LCM. The differentiated HL-60 phenotype, however, can clearly be expressed in the absence of spermidine when using TPA as inducer. These findings might therefore suggest that spermidine and DNA replication are required for induction of HL-60 differentiation with certain inducers other than TPA, while disruption of ongoing DNA synthesis with S-phase-specific agents (4) is not sufficient to inhibit appearance of the differentiated phenotype.

REFERENCES

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