L-Ethionine as an Inducer of Differentiation in Human Promyelocytic Leukemia Cells (HL-60)\(^1\)

Naomi Mendelsohn,\(^2\) Josef Michl,\(^3\) Harriet S. Gilbert, George Acs, and Judith K. Christman\(^4\)

Departments of Biochemistry [N. M., G. A., J. K. C.], Hematology [H. S. G.], and Pediatrics [N. M., G. A., J. K. C.], Mount Sinai School of Medicine, City University of New York, New York 10029, and The Rockefeller University, New York, New York 10021 [J. M.]

ABSTRACT

The methionine analog, L-ethionine, induces morphological and biochemical changes in cultured HL-60 cells which are indicative of myeloid maturation. After 3 to 5 days of growth in the presence of L-ethionine, the majority of cells have enhanced phagocytic ability. The percentage of cells in the culture which bear complement receptors and which can respond to 12-O-tetradecanoylphorbol-13-acetate with respiratory burst activity increases more than 3-fold. Since the cells fail to become adherent and lose nonspecific esterase activity, we conclude that L-ethionine, like dimethyl sulfoxide, induces granulocytic differentiation of HL-60 cells.

INTRODUCTION

Ethionine, the ethyl analog of the essential amino acid, methionine, has been most extensively studied for its effects on liver metabolism, since prolonged feeding of this compound induces hepatic carcinoma in rats (7, 23). A number of lines of evidence suggest that L-ethionine or one of its metabolites can also influence the expression of differentiated function in a variety of cell types. Ethionine administration can be linked to morphological changes in liver and pancreas (9, 27), rapid increases in \(\alpha\)-fetoprotein and progesterone levels in the serum (14, 26), and increases in the level of a number of enzymes in the liver (6, 31).

This compound also has profound effects on cells in culture. We have previously reported that L-ethionine can act as an inducing agent for erythroid maturation of Friend erythroleukemia cells (2). We report here that 2 \(\mu\)M L-ethionine added to the growth medium of cultured human promyelocytic leukemia cells (HL-60) induces expression of normal functional characteristics of mature myeloid cells within 3 to 5 days. An examination of morphological, histochemical, and biochemical parameters revealed no qualitative differences between Me\(_2\)SO\(^5\)-induced and ethionine-induced maturation.

MATERIALS AND METHODS

Reagents. Media and serum were from Grand Island Biological Co. (Grand Island, N. Y.). Tissue culture ware came from Falcon Plastics (Oxnard, Calif.). Multispec microscope slides were from C. A. Hendley and Co. (Essex, England). Ferricytochrome c, superoxide dismutase, and nitro blue tetrazolium were from Sigma Chemical Co. (St. Louis, Mo.). TPA was from Consolidated Midland Corp. (Brewster, N. Y.). Latex particles were from Dow Diagnostics (Indianapolis, Ind.). [1-\(^{14}\)C]Glucose and scintillation fluor were purchased from New England Nuclear (Boston, Mass.).

Cells. The human leukemic cell line, HL-60, was the gift of Dr. S. J. Collins, National Cancer Institute, Bethesda, Md. The cells are maintained in suspension culture in RPMI-1640 supplemented with 20% heat-inactivated FBS. Induced cells were obtained by seeding HL-60 cells at 2.5 \(\times\) 10\(^5\)/ml in growth media in the presence of 2 \(\mu\)M L-ethionine or 1.15% Me\(_2\)SO and continuing culture for the indicated times.

Measurement of CO\(_2\) Release. Cells were washed in 0.9% NaCl solution and resuspended at 5 \(\times\) 10\(^5\)/ml in glucose-free Earle’s balanced salt solution buffered at pH 7.2 with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and supplemented with 10% dialyzed FBS and 0.2 \(\mu\)Ci of [1-\(^{14}\)C]glucose (specific activity, 5.7 mCi/mmol). TPA was added to 1.66 \(\times\) 10\(^{-8}\) M as indicated. CO\(_2\) released by 1 ml of cell suspension during 1 hr incubation at 37\(^\circ\) was trapped in a Hyamine-soaked filter paper in a glass well suspended above the incubation mixture (33), the reaction was terminated by injection of 100 \(\mu\)l of concentrated sulfuric acid, and incubation was continued for 15 min at 37\(^\circ\) to ensure that all dissolved CO\(_2\) was released from the acidified medium.

Measurement of Superoxide Anion Production. The ratio of superoxide anion release by HL-60 cells was determined by measurement of ferricytochrome c reduction in the presence of intact cells. Two \(\times\) 10\(^5\) cells were suspended in 1 ml of Earle’s balanced salt solution (pH 7.2) containing 80 nmol of ferricytochrome c and, where appropriate, 1.66 \(\times\) 10\(^{-11}\) mol of freshly diluted TPA with or without 50 \(\mu\)g of superoxide dismutase, and incubated for 30 min at 37\(^\circ\). The reaction was stopped by chilling in an ice bath, and the cells were pelleted by centrifugation at 8000 \(\times\) g for 1 min in an Eppendorf microcentrifuge. The absorbance was determined, and the concentration of reduced cytochrome c was calculated, using the equation \(E_{550}\) = 2.1 \(\times\) 10\(^4\) \(\text{M}^{-1}\) \(\text{cm}^{-1}\) (16). Superoxide anion generated is expressed as nmol/10\(^6\) cells/hr.

The percentage of cells producing superoxide anion was determined by counting the number of cells capable of reducing nitro blue tetrazolium dye. Cells were suspended at 2 \(\times\) 10\(^5\)/ml in RPMI-1640 medium supplemented with 20% FBS and incubated for 20 min with an equal volume of 0.2% nitro blue tetrazolium dissolved in 0.15 \(\mu\)l NaCl/0.015 M sodium phosphate, pH 7.4, in the presence and absence of 1.66 \(\times\) 10\(^{-8}\) M freshly diluted TPA. At least 200 cells from each assay tube were counted to determine the percentage of cells containing intracellular reduced blue-black formazan deposits.
Measurement of Phagocytic Index. Ingestion of latex particles by HL-60 cells was measured as essentially described by Michl et al. (19). Washed 1.01-μm latex particles were added in a ratio of 40:1 to a 1-ml cell suspension (5 x 10⁶ cells/ml) RPMI-1640, 20% FBS, in a 12 x 75 mm Falcon tube. After a 1-hr incubation at 37°, the mixture was diluted with 5 ml medium, and cells were collected by centrifugation at 60 x g for 10 min. The supernatant containing noningested particles was removed, and the cells were gently resuspended in fresh medium and centrifuged onto polylysine-coated coverslips (20). Fixation with 2.5% glutaraldehyde was performed as described in Ref. 18. At least 100 cells were examined by phase microscopy, using a X100 oil immersion lens to evaluate the number of cells with ingested particles and the number of particles per cell.

Assay for Surface Receptors. Surface receptors for the Fc portion of IgG were determined by a human erythrocyte antibody-rosetting technique (12, 32). Human erythrocytes positive for Rh antigen D (Rh:1) were incubated with the serum of a patient, Heyman, kindly provided by Dr. Richard Rosenfeld, Mount Sinai Hospital, New York, N. Y., which has a high anti-D-IgG antibody titer, to form antibody-coated erythrocytes (12). Rosette formation was assayed by incubating 50 μl of HL-60 cells (4 x 10⁶/ml) in Hanks’ balanced salt solution with an equal volume of the Heyman antibody-coated erythrocytes (2 x 10⁶/ml) in plastic tubes. After gentle agitation for 1 hr at 37°, the mixture was centrifuged at 150 x g for 5 min and gently rocked to disrupt the cell pellet, and aliquots were transferred to wells on a multipotential slide to enumerate rosettes.

Surface receptors for complement were assayed with human erythrocytes exposed to fresh serum (11). Erythrocytes from 50 μl of whole blood were suspended in 0.2 ml of fresh serum plus 0.9 ml of 10% sucrose, pH 6.5, and allowed to remain at room temperature for 30 min. The resulting complement-coated erythrocytes were adjusted to a concentration of 2 x 10⁸ cells/ml, 50 μl were mixed with an equal volume of HL-60 cell suspension (4 x 10⁶ cells/ml) and incubated for 1 hr at room temperature, and rosettes were enumerated.

Measurement of NSE and Peroxidase. The percentage of cells possessing β-naphthyl esterase activity was determined by automated cytochemical analyses of cell suspensions. Discriminator settings were optimized for detection of esterase-positive human monocytes (13, 22).

% of NSE-positive cells = (% of cells esterase positive – % of cells esterase positive in the presence of 10 mm NaF)

The percentage of cells with peroxidase was scored by automated cytochemical analysis after staining with 4-chloronaphthol, using discriminator settings optimized for detection of peroxidase-positive human leukocytes (13, 22).

RESULTS

As we have previously reported (18), Me₂SO-induced granulocytic maturation of HL-60 cells is associated with the appearance of a TPA-sensitive NADPH oxidase. Activation of this enzyme in the presence of TPA results in a marked increase in the rate with which the cells catabolize glucose via the hexose monophosphate shunt. Thus, measurement of TPA-stimulated release of CO₂ from C-1 of glucose provides a convenient quantitative measure of maturation of the cells.

The data in Table 1 show that HL-60 cells grown in the presence of L-ethionine also display a TPA-stimulated increase in hexose monophosphate shunt activity which is much larger than that of untreated cells. The extent of this increase in responsiveness to TPA is dependent on the concentration of L-ethionine in the growth medium and, at higher concentrations, can approach 75% of that observed in cells grown for the same period of time in the presence of Me₂SO. At all concentrations tested, L-ethionine slowed the growth of the cells, with cells cultured in the presence of 4 mM L-ethionine showing little net increase in viable cell number over a 5-day period. However, cells grown in 2 mM L-ethionine remained viable (~85%) for at least 7 days and developed increased responsiveness to TPA (Chart 1a) with a time course similar to that which we observed for cells grown in the presence of Me₂SO (18). While maturation measured as release of CO₂ from C-1 of glucose in the presence of TPA could be enhanced by increasing ethionine concentrations between 2 and 4 mM, this gain was offset by poor growth and marked loss of viability between 3 and 6 days in culture (data not shown). On the basis of these results, we chose to use 2 mM L-ethionine for all subsequent experiments. It can be seen in Chart 1b that L-ethionine need not be present for the entire culture period in order to exert its effect. Exposure (24 to 48 hr) to L-ethionine at the onset of a 7-day culture period is sufficient to increase the TPA-stimulated hexose monophosphate shunt activity of HL-60 cells significantly in comparison to untreated cells (activity measured at Day 7 of culture). Four days of exposure to L-ethionine is comparable in its effect of exposing the cells to ethionine for the full 7 days, while 5 days of exposure actually seems more effective in evoking TPA-stimulated activity. This latter result may indicate

<table>
<thead>
<tr>
<th>Addition to culture medium</th>
<th>Viable cells (percent on Day 5)</th>
<th>Growth rate (percent untreated culture)</th>
<th>TPA-stimulated CO₂ release (cpm/10⁶ viable cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Ethionine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 mM</td>
<td>42 ± 10⁶</td>
<td>11 ± 1</td>
<td>7,870 ± 850</td>
</tr>
<tr>
<td>3.2 mM</td>
<td>73 ± 8</td>
<td>28 ± 5</td>
<td>13,200 ± 2,425</td>
</tr>
<tr>
<td>2.6 mM</td>
<td>78 ± 5</td>
<td>30 ± 6</td>
<td>12,750 ± 1,150</td>
</tr>
<tr>
<td>2.4 mM</td>
<td>90 ± 5</td>
<td>36 ± 3</td>
<td>11,500 ± 980</td>
</tr>
<tr>
<td>2 mM</td>
<td>95 ± 5</td>
<td>45 ± 3</td>
<td>11,400 ± 750</td>
</tr>
<tr>
<td>1 mM</td>
<td>96 ± 5</td>
<td>52 ± 2</td>
<td>6,940 ± 800</td>
</tr>
<tr>
<td>Me₂SO (1.15%)</td>
<td>96 ± 3</td>
<td>62 ± 3</td>
<td>17,500 ± 1,500</td>
</tr>
<tr>
<td>None</td>
<td>95 ± 3</td>
<td>100</td>
<td>5,400 ± 450</td>
</tr>
</tbody>
</table>

*Cells were seeded at 2.5 x 10⁶/ml in RPMI 1640 supplemented with 10% FBS, and the indicated additions were incubated at 37° in a moist 5% CO₂ chamber for 5 days.

b Cell number and viability (trypan blue exclusion) were determined daily.

c Growth rate = 100 x cell density of treated culture

cell density of untreated culture

don Day 5. Viable cell number was determined daily. For all cultures containing less than 3 mM L-ethionine, viable cell number increased daily during the 5-day culture period. At L-ethionine concentrations >3 mM, viable cell density declined no more than 10 to 15% between Days 4 and 5. Untreated cultures were still in the logarithmic stage of growth on Day 5 and had reached a cell density of 1.1 x 10⁶/ml.

On Day 5, cells were harvested and resuspended at 5 x 10⁶ viable cells per ml for determination of ¹⁴CO₂ release from [1-¹⁴C]glucose, as described in Materials and Methods. Background ¹⁴CO₂ release in the absence of TPA has been subtracted. This value is 550 ± 40 cpm.

* Mean ± S.E. of 3 separate experiments.
that removal of ethionine for the final 2 days of culture reduces some cumulative toxic effect of the compound.

Morphological and functional examination of the cells grown in L-ethionine confirmed our initial supposition that development of TPA-stimulable hexose monophosphate shunt activity indicated induction of granulocytic maturation of HL-60 cells. After 5 days of growth in the presence of 2 mM L-ethionine, the percentage of mature myeloid forms (myelocytes, metamyelocytes, banded neutrophils, and segmented neutrophils) increases to 80 to 90%, compared with 15% or less in untreated cultures (Fig. 1). During this period of exposure to L-ethionine, the cells increase their ability to phagocytize latex particles [phagocytic index = 10.4 versus 2.87 for untreated cells (Table 2)]. As shown in Table 3, they acquire the expected TPA-sensitive NADPH-oxidase, as indicated by an increase both in the rate of superoxide anion production and in the percentage of cells capable of superoxide anion production in the presence of TPA. Maturation was also accompanied by an increase in the percentage of cells bearing complement receptors and a decrease in the percentage of NSE-positive cells. Neither Me2SO nor ethionine-induced maturation markedly affects the levels of myeloperoxidase or high-affinity Fc receptor-bearing cells in the population (Table 3). Thus, in every respect that we have examined, ethionine-treated cells resemble HL-60 cells undergoing granulocytic differentiation in the presence of Me2SO.

DISCUSSION

Since Collins et al. (3) first described the ability of Me2SO to induce HL-60 cells to undergo morphological changes indicative of myeloid maturation, these cells have been studied by several groups who have shown that growth in the presence of Me2SO also induces the cells to develop many of the functional and biochemical characteristics of mature circulating granulocytes (4, 10, 18, 21, 29). The cells acquire the ability to ingest

Chart 1. TPA-sensitive hexose monophosphate shunt activity of HL-60 cells as a function of time of exposure to L-ethionine. a, cells grown in the presence (□, □) or absence (○, ○) of 2 mM L-ethionine assayed for CO2 release from [1-14C]glucose after the indicated days in culture. □, □, 1.68 × 10^{-4} M TPA present in assay mixture; ○, ○, minus TPA. b, cells grown in the presence of 2 mM L-ethionine for the indicated number of days, washed free of the compound, and resuspended in ethionine-free medium. Assay for CO2 release was performed after the cells had been in culture for a total of 7 days. Data shown are for CO2 release in the presence of 1.68 × 10^{-4} M TPA. CO2 release by cells grown for 7 days in the absence of L-ethionine has been subtracted (+TPA, 4500 cpm/5 × 10^6 cells/30 min) to obtain the values shown, which represent the mean of 3 separate determinations. All details as in Table 1.

Fig. 1. Typical fields from a Wright-Giemsa-stained preparation at × 1250 are shown. a, untreated HL-60 cells cultured 5 days after seeding in fresh medium. b, HL-60 cells cultured for 5 days in the presence of 2 mM L-ethionine.
L-Ethionine as Inducer of Differentiation

Ingestion of latex particles by HL-60 cells grown in L-ethionine and Me2SO for 5 days

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>% of cells with indicated no. of particles</th>
<th>Av. no. of particles/cell</th>
<th>Pi*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethionine (2 mM)</td>
<td>&gt;20: 38 ± 5 10–20: 14 ± 6 1–9: 36 ± 3 0: 10 ± 2</td>
<td>11.6 ± 3.5 10.4</td>
<td></td>
</tr>
<tr>
<td>Me2SO (1.15%)</td>
<td>&gt;20: 36 ± 10 10–20: 24 ± 10 1–9: 36 ± 5 0: 4 ± 1</td>
<td>14.2 ± 2.3 13.6</td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>&gt;20: 2 ± 1 10–20: 6 ± 2 1–9: 74 ± 10 0: 18 ± 5</td>
<td>3.5 ± 1.8 2.87</td>
<td></td>
</tr>
</tbody>
</table>

* Pi, phagocytic index of percentage of cells ingesting latex particles × average number of particles per cell.

Table 3

Comparison of effects of L-ethionine and Me2SO on HL-60 cells

All assays were performed on cells cultured for 5 days.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>% of cells reducing nitro blue tetrazolium</th>
<th>% of cells bearing Fc rosettes</th>
<th>% of cells bearing C rosettes</th>
<th>% of cells staining for NSE</th>
<th>% of cells staining for myeloperoxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Ethionine (2 mM)</td>
<td>146 ± 14 27 ± 5 13 ± 2 53 ± 9 10 ± 3 83 ± 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Me2SO (1.15%)</td>
<td>236 ± 11 56 ± 8 15 ± 4 66 ± 12 12 ± 2 86 ± 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions</td>
<td>38 ± 10 10 ± 2 18 ± 2 11 ± 2 40 ± 5 92 ± 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Results are for measurements in the presence of 1.66 × 10^-8 M TPA and represent changes which are inhibitable by superoxide dismutase. In the absence of TPA, cells produced 13 ± 4 mmol/10⁶ cells/hr, regardless of prior culture conditions.

** Results are for measurements in the presence of 1.66 × 10^-8 M TPA. In the absence of TPA, the percentage of nitro blue tetrazolium-positive cells was less than 1%.

It should be noted that, while L-ethionine is a highly active inducer, mediating its effect on maturation of both HL-60 cells and mouse erythroleukemia cells at concentrations 1 to 2% of those required for Me2SO, it is generally less efficient than is Me2SO in terms of the percentage of the population attaining the mature phenotype. It is not yet clear whether this indicates a heterogeneity of the population with regard to ability to respond to L-ethionine as an inducer or that other metabolic effects of L-ethionine interfere with full development of the mature phenotype in some cells. Although L-ethionine has the capacity to deplete the ATP pool under conditions of methionine starvation (28), under our experimental conditions with 0.1 mM L-methionine present, the ATP pool is not depleted and macromolecular synthesis rates are sufficient to maintain cell growth and viability over a 5– to 7-day culture period. This does not preclude the possibility that prolonged exposure to L-ethionine affects synthesis of specific macromolecules. The experiments shown in Chart 1b hint that such a toxic effect might occur in HL-60 cells.

The mechanisms by which L-ethionine and other inducing agents trigger differentiation are as yet unknown. Mouse erythroleukemia cells appear to require 1 to 2 cell divisions in the presence of an inducing agent. With a 24-hr exposure, a fraction of the population becomes committed to differentiation and no longer requires the inducing agent in order to complete the differentiation process. Between 48 and 72 hr of exposure are required for all of the cells to become committed (8). The results in Chart 1b indicate that a similar commitment process occurs when HL-60 cells are grown in the presence of L-ethionine, with a small fraction of the cells becoming committed within 24 to 36 hr, and all of the cells with the potential for induction becoming committed between 4 and 5 days in culture. Our finding that L-ethionine, a compound which in the form of S-adenosylmethionine can inhibit a variety of methyltransferases, is an inducer of differentiation suggests that inhibition of methyl transfer may be involved.

Numerous suggestions have been made as to how methylation of specific sites in DNA (or lack thereof) could be heritable and could thus serve to regulate gene activity during differentiation (15, 24). Recent reports have associated the presence of unmethylated CCGG sequences with active globin gene expression in the chicken (17) and with expression of the Herpesvirus saimiri genome (5). Such specific unmethylated sites have been demonstrated in other genes, but without compelling evidence for an effect on gene activity (1, 30). The results reported here reinforce the linkage between inhibition of methylation and differentiation by showing that L-ethionine can act as an inducer of maturation in more than one species and cell type. Work is now in progress in our laboratory to determine whether methylation of DNA in HLA-60 cells is affected in the same way during differentiation as is mouse erythroleukemia cell DNA.

References


I-Ethionine as an Inducer of Differentiation in Human Promyelocytic Leukemia Cells (HL-60)

Naomi Mendelsohn, Josef Michl, Harriet S. Gilbert, et al.


Updated version   Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/40/9/3206

E-mail alerts   Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions   To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions   To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/40/9/3206. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.