Essential Amino Acid Deprivation Induces Monocytic Differentiation of the Human HL-60 Myeloid Leukemia Cell Line

By Kim E. Nichols and J. Brice Weinberg

In this study we examine the effects of amino acid deprivation on the growth and differentiation of the human HL-60 myeloid leukemia cell line. The HL-60 cell line was chosen for study because of its ability to differentiate along either a granulocytic or monocytic pathway under appropriate culture conditions. Differentiation was determined by changes in cell morphology, nonspecific esterase (NSE) content, hydrogen peroxide (H$_2$O$_2$) production, and expression of the cell surface differentiation antigens LeuM3 (CD14) and OKM1 (CD11). Using a model system in which HL-60 cells were cultured in medium that selectively lacked one amino acid (AA), it was seen that deprivation of HL-60 cells for essential (but not nonessential) AAs results in decreased cell growth and viability and in differentiation of 30% to 60% of the surviving population of cells specifically along the monocytic pathway. This differentiation is irreversible as well as time- and dose-dependent. Culture of HL-60 cells in essential AA-deficient medium potentiated the differentiative effects of recombinant human interferon-gamma (IFN-γ), recombinant human tumor necrosis factor (TNF), and dihydroxyvitamin D$_3$ (D$_3$), all of which have previously been shown to induce monocytic differentiation of HL-60 cells. Differentiated cells had decreased DNA and RNA synthesis, but protein synthesis was unchanged compared with control cells. The protein synthesis inhibitor cycloheximide prevented differentiation, indicating the necessity of protein synthesis in this process. Cell cycle analysis revealed that an increased proportion of cells cultured in AA-deficient medium was arrested in G0-G1 (80%) and 50% for AA-deficient and control cells, respectively. These results suggest that alterations of AA metabolism and subsequent perturbations in DNA and RNA synthesis may be important in initiating differentiation or in augmenting cytokine-induced differentiation of HL-60 cells into more mature, nonreplicating, monocyte-like cells.

The Human Myeloid leukemia cell line HL-60 has proved to be a useful model for investigating hematopoietic cell differentiation. The cell line, derived from a patient with acute nonlymphocytic leukemia, is remarkable for its ability to grow in vitro without the addition of growth factors and for its ability to differentiate when cultured with various inducers of differentiation.

In culture, these cells grow as a homogeneous population of immature myeloid cells, but under appropriate conditions they may be induced to differentiate phenotypically and functionally into cells that resemble either monocytes, granulocytes, multinucleated giant cells, or eosinophils. While several agents have been identified that can induce differentiation of HL-60 cells, little is known concerning the mechanisms involved in differentiation and their regulation. Such mechanisms are complex and are felt to involve events at the transcriptional, translational, and post-translational levels.

Previous investigation has shown that interference with polyamine metabolism affects the growth and differentiation of a number of human and murine leukemia cell lines in vitro. Amino acid (AA) metabolism is closely related to polyamine metabolism, and we postulated that manipulations in amino acid metabolic pathways might differentiate human leukemia cells and provide insight into the mechanisms involved in the differentiation process. To investigate this phenomenon, cells of the HL-60 cell line were cultured in medium that was deficient in one AA. Most experiments were done using medium that lacked arginine (ARG) because this AA is closely tied to polyamine metabolism and, in particular, to the synthesis of ornithine, which then may be converted to putrescine, spermidine, and spermine. In addition, ARG has been shown to play an important role in leukocyte maturation and function. After four to seven days of culture, cells were assessed for changes in growth and differentiation.

Results show that HL-60 cells deprived of an essential AA have a decrease in proliferation paralleled by an increase in cell differentiation along a monocytic (but not neutrophilic) pathway. On a cellular level there were changes indicating that differentiation is accompanied by a perturbation in DNA and RNA synthesis with preservation of protein synthesis.

MATERIALS AND METHODS

Cell lines. The HL-60 cell line was obtained from the American Type Culture Collection, Rockville, MD. Cells were routinely passed at a concentration of 1.0 to 2.5 × 10$^5$ cells/mL in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sterile Systems, Logan, UT), 100 U/mL penicillin and 100 mg/mL streptomycin. Cell passage number was below 50 for all experiments. Less than 1% of cells were nonspecific esterase (NSE) positive at the start of all cultures.

Culture conditions. For experiments, cells were recovered from routine growth medium by centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended at a density of 2.5 × 10$^5$ cells/mL in RPMI 1640 that was selectively deficient for one AA (Selectamine Kit, GIBCO). This medium was supplemented with penicillin/streptomycin, as in routine cultures, and with 10% FBS previously dialyzed against PBS for three days to remove amino acids. Dialysis tubing of 2,000 daltons molecular weight (mol/wt) cut-off was used to allow retention of insulin, transferrin, and other serum-associated growth factors required for the proliferation of HL-60 cells. Cultures were maintained at 37°C with 100% humidity.

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and 5% CO₂ for one to seven days. Additional agents were routinely added to cultures at initiation of incubation. Prior to all assays, cells were harvested by centrifugation, washed three times with PBS, and cell number and viability were determined using trypan blue exclusion.

**Hydrogen peroxide production.** Cells were assayed for the ability to produce hydrogen peroxide (H₂O₂) in the presence or absence of 200 nmol/L phorbol myristate acetate (PMA) by using horseradish peroxidase, phenol red, and a microtiter plate reader (spectrophotometer) as described previously.¹² In all experiments H₂O₂ assays were done in triplicate, and H₂O₂ production was expressed as nmol H₂O₂ produced/1 x 10⁶ cells/h.

**Morphology and NSE content.** Cells were collected onto glass slides using a cytocentrifuge (Shandon Southern, Astmoor, England). When dry, the slides were stained with Wright's and alpha-naphthyl butyrate esterase (NSE) stains as described previously.¹⁴ In all experiments the percentage of cells that were NSE positive was determined by counting in a blinded fashion three separate fields per cytopsin preparation.

**Cell-surface antigen studies.** Cells were processed for indirect immunofluorescence and analysis on an EPICS V fluorescent cell analyzer (Coulter, Hialeah, FL) as described previously.¹³ Nonviable cells were gated out in terms of overall size and fluorescence intensity and therefore were excluded from analysis. Antibodies detecting HLA-Dr and LeuM3 (CD14) were purchased from Becton Dickinson (Mountain View, CA) and OKM1 (CD11) from Ortho Diagnostics (Raritan, NJ).

**Assays for protein, DNA, and RNA synthesis.** HL-60 cells were cultured at 1 x 10⁵ cells/microtiter well in 0.2 mL medium, including all additives. Next, either 1 μCi [methyl-³H]thymidine (2 Ci/mmol, New England Nuclear, Boston, MA), [5,6-³H]uridine (2 Ci/mmol, Amersham, Arlington Heights, IL) or L-[4,5-³H]leucine (57 Ci/mmol, Amersham) was added to each well. After either 18, 18, or 4 hours of culture, respectively, cells were harvested onto glass fiber paper with a multiple automated sample harvester (Becton Dickinson, Vineland, NJ). For all assays, cells were harvested with PBS, followed with 10% trichloroacetic acid, methanol, and distilled H₂O. The filter discs were counted in liquid scintillant (Research Products International, Mount Prospect, IL) on a scintillation counter.

**Cell cycle phase analysis.** One milliliter cold 70% ethanol in PBS was added to a pellet of 1 x 10⁶ cells. The pellet was vortexed and incubated on ice for 20 minutes. Next, the fixed cells were washed twice with cold PBS and resuspended in 0.5 mL of a 0.00625% (wt/vol) propidium iodide solution containing 0.002% (wt/vol) RNase. Cells were analyzed for nuclear staining and DNA content with an EPICS V fluorescent cell sorter as previously described.¹⁶ As in the cell surface antigen studies, nonviable cells were gated out and excluded from analysis.

**Materials.** Purified recombinant human interferon-gamma (IFN-γ) was obtained from Genentech (San Francisco), purified recombinant human tumor necrosis factor (TNF) from Cetus Corporation (Emeryville, CA), and D₂ from Hoffman LaRoche (Nutley, NJ). All other reagents, unless otherwise specified, were from Sigma Chemical Co (St. Louis).

**RESULTS**

**Cell growth.** HL-60 cells deprived of ARG or another essential (but not nonessential) AA show a decrease in proliferation and in viability. In general, after four days of culture in ARG-deficient medium, total cell number recovered was 30% of the initial number plated, whereas control cell number was 300% to 500% of the initial number plated. Viability of the recovered cells was >98% for control and 30% to 50% for ARG-deprived cells. As shown in Fig 1, the inhibition of proliferation in ARG-free medium could be prevented in a dose-dependent fashion by adding ARG back to the culture medium at initiation of culture. It was seen that a final concentration of approximately 10 to 25 mmol/L ARG was required to maintain cell growth and proliferation and to prevent differentiation.

**Cell morphology and cytochemistry.** Examination of cytopsin preparations stained with Wright's stain revealed that ARG-deprived HL-60 cells were larger and more vacuolated than control cells and had increased ruffling of the plasma membrane (Fig 2). The change in morphology suggested differentiation of ARG-deprived cells along a monocytic pathway. These cells, however, were not adherent to the plastic culture dishes, as are more mature monocytes or HL-60 cells induced to differentiate into monocytes by phorbol diester.¹² ¹₈ ¹₉ To assess further the degree of differentiation, ARG-deprived cells were examined for NSE content, which has previously been identified as a marker or monocytic differentiation.²⁰ As shown in Fig 3, after four to seven days of ARG deprivation, 30% to 70% of viable cells exhibited increased NSE content. Parallel experiments in which ARG-deprived cells were processed with Ficoll-Hypaque (FH) to remove nonviable cells prior to NSE staining gave equivalent results. Although 0% to 1% of the initial population of HL-60 cells was positive for NSE, the increase in the number of cells positive for NSE after ARG deprivation was too great to have resulted merely from selection of an initially positive population. Instead, ARG deprivation induced differentiation of HL-60 cells along a monocytic pathway. The time course of NSE induction was gradual, with maximal NSE content present at seven days (Fig 3). When ARG-deprived cells were washed, recultured in complete medium for five days, and reanalyzed, it was found that differentiation was irreversible (data not shown). As with cell growth, the differentiating effects of ARG deprivation could be prevented in a dose-dependent fashion by adding ARG to the culture medium at initiation of culture (Fig 4).

**Fig 1.** Growth curves of HL-60 cells in medium with different concentrations of ARG. ARG was added at initiation of culture. Viable cell number was determined by trypan blue exclusion and is expressed as total viable cells/culture dish. Values are from one experiment that is representative of six experiments performed.
Fig 2. Morphological and cytochemical changes in ARG-deprived HL-60 cells. Cells were incubated for four days in complete and in ARG-deficient medium, then harvested and studied. Cells positive for NSE are grey-black in appearance. (a) Control cells. Wright’s stain (original magnification x 1,000). (b) Cells cultured with 0 ARG. Wright’s stain (original magnification x 1,000). (c) Control cells. NSE stain (original magnification x 400). (d) Cells cultured with 0 ARG. NSE stain (original magnification x 400).

Hydrogen peroxide production. It has previously been shown that the differentiation of HL-60 cells to more mature myeloid or monocytic cells is marked by the acquisition of the ability to produce reactive oxygen species, specifically H$_2$O$_2$ and superoxide, in response to various stimuli including PMA.$^{2,3}$ Cells deprived of ARG showed an increase in H$_2$O$_2$ production that paralleled the expression of NSE in a dose- and time-dependent fashion (Figs 3 and 4).

Specificity of the response. Experiments were designed to examine the effects of starvation for other essential and
HL-60 CELL DIFFERENTIATION

Fig 3. Time course of NSE induction and H2O2 production in ARG-deprived HL-60 cells. (Top) NSE induction. (Bottom) H2O2 production. Values represent the mean ± SD from triplicates within one experiment that is representative of three performed.

nonessential AA on HL-60 cell growth and differentiation. As shown in Table 1, starvation for a number of other essential AA resulted in decreased cell proliferation and viability as well as in induction of H2O2 production and NSE content. In contrast, starvation for a nonessential AA (eg, aspartate) caused no change in cell growth or differentiation.

Expression of differentiation antigens. When examined for the expression of a panel of differentiation antigens, it was found that after four days of culture in ARG-deficient medium, 60% to 70% of cells expressed the monocyte differentiation antigens defined by anti-Leu M3 and anti-OKM122 (Table 2). These results correlate well with the percentage of ARG-deprived cells that are induced to express NSE. In addition to an increased percentage of cells showing acquisition of monocyte differentiation antigens, the differentiated cells also showed an increased density of antigen per cell as indicated by the increase in mean fluorescence intensity.

Table 1. Effects of AA Deprivation on Growth and Differentiation of HL-60 Cells

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cell No. (Millions)</th>
<th>Viability (% viable)</th>
<th>H2O2 (nmol/h)</th>
<th>NSE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.0</td>
<td>95</td>
<td>0.6</td>
<td>0.3</td>
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<tr>
<td>0 ARG</td>
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<td>51</td>
<td>2.9</td>
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<tr>
<td>0 ASP</td>
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<td>0.2</td>
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</tr>
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<tr>
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<td>28</td>
<td>3.5</td>
<td>4.7</td>
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</table>

Culture of HL-60 cells in essential AA-deficient medium decreases growth and induces differentiation. Cells were cultured in medium lacking one AA for four days and then were harvested and assayed for growth and differentiation. Values represent the results from one experiment that is representative of two performed.
DNA, RNA, and protein synthesis. To further characterize the events involved in the differentiation of HL-60 cells after four days of ARG deprivation, we measured the level of DNA, RNA, and protein synthesis in control and ARG-deprived cells. As shown in Fig 5, incorporation of tritiated thymidine and uridine decreased to approximately 12% and 25% of control levels, respectively, whereas incorporation of tritiated leucine remained unchanged as compared with control levels. These data suggest that there is a decrease in the ability of ARG-deprived cells to make DNA and RNA but that there is preservation of protein synthesis during differentiation. This maintenance of protein synthesis occurs despite the lack of a major exogenous source of ARG.

Effect of protein synthesis inhibition on ARG-deprived HL-60 differentiation. To further examine the importance of protein synthesis during differentiation, cells were examined for differentiation in the presence or absence of cycloheximide (1 μg/mL). This protein synthesis inhibitor is an irreversible inhibitor of the enzyme, ribosomal peptidyl transferase. Culture of cells in ARG-deficient medium with cycloheximide resulted in decreased cell growth and viability as well as in reduced induction of NSE and H₂O₂-producing ability (Fig 6). Cycloheximide alone did not induce differentiation. Parallel experiments demonstrated that at this concentration cycloheximide did not affect cell viability but inhibited proliferation by 30% to 50% as compared with control cells, and protein synthesis by 85%. These results suggest that the differentiation induced by culture in ARG-deficient medium is dependent on new protein synthesis.

Additive effects of AA starvation and treatment with IFN-γ, D₃, and TNF. It is known that culture of HL-60 cells with recombinant IFN-γ, TNF, or D₃ induces monocytic differentiation as well as formation of NSE-positive multinucleated giant cells. In our experiments starvation for ARG potentiated the effects of IFN-γ, TNF, and D₃ in inducing monocytic differentiation as measured by increased NSE content and enhanced H₂O₂ production. The
potentiation was additive and would occur when cells were either cultured in ARG-deficient medium with inducer (Fig 7) or cultured first in ARG-deficient medium and then converted to conventional complete medium with the inducer (data not shown).

**Cell cycle phase analysis.** Other investigators have demonstrated that there are critical stages in the cell cycle in which tumor cells become arrested when they are induced to differentiate. For example, Boyd and Metcalf have shown that butyrate-induced terminal differentiation of HL-60 cells is accompanied by an arrest in the G1 phase of the cell cycle, suggesting that the switch to differentiation pathways occurs either late in G2-M or early in G1. To further characterize and understand the events that occur during essential AA deprivation, cell-cycle analysis was performed. As shown in Fig 8, ARG deprivation resulted in an increase in the percent of cells in G0-G1 (81 ± 34 for control cells) and a decrease in the percent of cells in S phase (10 ± 32 for control cells). The results correlate with the decrease in proliferation and reduced incorporation of tritiated thymidine seen in parallel experiments and suggest an integral role for essential AA in growth regulation and cell replication.

**Effect of AA deprivation on growth and differentiation of other leukemia cell lines.** To examine the effect of AA starvation on the growth and differentiation of other leukemia cell lines, cells from the KG-1 (myeloblastic), U937 (monoblastic), and K562 (erythroleukemia) cell lines were cultured in ARG-deficient medium and analyzed as described previously. Cell growth in ARG-deficient medium was inhibited by 97%, 90%, and 98% over control cells, respectively, with viability of remaining cells being 40%, 60%, and 29% over control cells, respectively, with viability of remaining cells being 40%, 60%, and 29%. ARG-deprived KG-1 cells produced 0.19, 0.24, and 0.66 nmol/h H2O2 (with control cells producing 0.07, 0.03, and 0.04 nmol/h). ARG-deprived KG-1 cells were 13% positive for NSE as compared with 1% of control cells. U937 cells are 100% NSE positive in control cultures, and this parameter of differentiation was not measured in this cell line. Evaluation of K562 cell differentiation by examination for hemoglobin synthesis showed no difference between control and essential AA-deprived cells (data not shown). These results indicate that several leukemia cell lines are sensitive to the growth inhibitory effects but show variable sensitivity to the differentiative effects of essential AA deprivation.
DISCUSSION

The human HL-60 myeloid leukemia cell line has proven to be useful in examining the processes involved in early leukocyte differentiation and its regulation. Use of the cell line in vitro and in vivo has helped to characterize the differences between granulocytic and monocytic differentiation and to identify agents that are capable of inducing differentiation along either of these pathways. Despite the fact that many agents have been identified that stimulate HL-60 differentiation, the precise molecular events involved in differentiation are not fully understood. Knowledge of these events would prove useful not only in understanding the hematopoietic process but also in designing chemotherapeutic agents beneficial in the treatment of leukemia. Ideally such agents would be employed with the intent of differentiating immature leukemia cells in vivo into more mature, nonreplicating, and perhaps functional cells.45

In this study we demonstrate a novel mechanism for regulating the growth and differentiation of HL-60 cells. Deprivation of an essential AA results in decreased proliferation and viability of HL-60 cells and in monocytic differentiation of a substantial percentage of the surviving population of cells. Prior investigations by Hunna et al42 have suggested that depleting culture medium of ARG with exogenous arginase results in differentiation of HL-60 cells and murine M-1 myeloblastic cells. In addition, Currie43 has shown that arginase may mediate the killing of various tumor cell lines by murine macrophages. Recent studies by Pilz et al44 have demonstrated that starvation of HL-60 cells for an essential AA results in differentiation.44 Although they termed this “myeloid” differentiation, their studies were not detailed enough to determine the lineage of differentiation. Our work clearly shows this differentiation to be monocytic in nature. With the “stress” of culture under these conditions, the immature myeloid leukemia cells appear to preferentially differentiate to the most phylogenetically primitive type of blood cell: the monocyte/macrophage.45

We note that differentiation induced by AA deprivation is irreversible as well as time- and dose-dependent. The time course is gradual, requiring four to seven days for expression; this is comparable to that required when conventional doses of other differentiating agents such as TNF, D3, and IFN-γ are used.13,28-31

The differentiation occurring in ARG-deficient cultures is accompanied by decreased DNA and RNA synthesis with preservation of protein synthesis. Although the decrease in DNA and RNA synthesis is in agreement with the findings of others who have studied hematopoietic cells in vitro,44,46,47 the maintenance of protein synthesis at control levels is unusual. These findings suggest that there are alternative mechanisms for procuring the essential AAs required for protein synthesis. One possible mechanism might be the degradation of existing proteins (eg, intracellular components or cellular components of nonviable cells) by the differentiated monocyte-like HL-60 cells. The fact that cycloheximide can inhibit differentiation of essential AA-deprived HL-60 cells supports the importance of maintaining protein synthesis during differentiation.

The mechanisms that underlie the induction of HL-60 cell differentiation by AA deprivation remain unknown at the present time; however, there are several possible explanations. First, others have suggested that perturbations in DNA synthesis, and subsequently in gene expression, are important in the differentiation of HL-60 cells. Yen et al48 have shown that PMA-induced monocytic differentiation of HL-60 cells is enhanced by interruption of DNA synthesis with hydroxyurea. In addition, Munroe et al49 have shown that adenine arabinoside, another agent capable of inhibiting DNA synthesis, induces monocytic differentiation of HL-60 cells. Although it is not yet understood how a decrease in DNA synthesis is related to differentiation, it is possible that alteration of oncogene expression may be involved. For example, McCachren et al50 have shown that during dibutyryl-cyclic adenosine monophosphate-induced differentiation of HL-60 cells, there is interruption of transcription, as well as reduced expression, of the c-myc oncogene.50 This oncogene product is a DNA binding protein that may be important in maintaining the immature phenotype in HL-60 and other human and murine leukemia cell lines. Second, inhibition of DNA replication may result in synthesis of aberrant forms of DNA, with certain segments being replicated at an increased rate.49,51,52 Thus additional copies of select DNA fragments may accumulate and lead to altered gene expression and, perhaps, the differentiated state. Third, it has been shown that culture of human lymphoblasts in AA-deficient medium results in decreased purine synthesis by both the de novo and salvage pathways,53 with synthesis of guanylates decreased more than synthesis of adenylates or pyrimidines. Various investigators have shown that a decrease in intracellular GDP and GTP pools is closely linked to terminal differentiation of HL-60 cells.53-57 Therefore it is possible that AA deprivation results in terminal differentiation of HL-60 cells by alteration or reduction of purine synthesis. Our preliminary studies, however, show that addition of exogenous guanosine to AA-deficient cultures does not prevent or reverse the effects of AA deprivation (data not shown).

The data presented here suggest a mechanism by which monocytic differentiation may be induced in myeloid leukemia cells by essential AA deprivation. This may reflect an important role for essential AAs in the regulation of normal and neoplastic cell growth. Currently our laboratory is investigating the effects of essential AA deprivation on the growth and differentiation of freshly isolated peripheral blood leukocytes from normal controls and leukemic patients. The clinical use of novel essential AA analogues or AA-degrading enzymes may be effective therapeutically in treating human nonlymphocytic leukemias by decreasing leukemia cell proliferation and inducing differentiation to more mature, functional cells. Furthermore, these treatments might potentiate the effects of other differentiating agents.

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