The Proto-Oncogene c-myc Blocks Myeloid Differentiation Independently of Its Target Gene Ornithine Decarboxylase

By Muthu Selvakumaran, Dan Liebermann, and Barbara Hoffman

Ornithine decarboxylase (ODC), a rate-limiting enzyme of polyamine biosynthesis, has been shown to be required for entry into and progression through the cell cycle and to be a transcriptional target of the proto-oncogene, c-myc. We show that ODC transcripts and enzyme activity are down-regulated following induction of myeloid differentiation, using M1 myeloblastic leukemic cells and normal cells from bone marrow (BM), and fail to be suppressed when c-myc expression is deregulated. In M1mycyc cells, when endogenous c-myc expression has been suppressed following stimulation by interleukin-6 (IL-6), treatment with estrogen and cycloheximide results in induction of ODC transcripts. These data demonstrate that ODC is a c-myc target gene in M1 cells. It was of interest to determine whether deregulated ODC expression would alter the myeloid differentiation program. To answer this question, M1-ODC cell lines constitutively expressing ODC were established. These can undergo terminal differentiation and growth arrest following IL-6 stimulation, exactly like parental M1 cells, demonstrating that deregulated ODC expression is not sufficient to block myeloid differentiation. Another question to be answered was whether ODC expression is necessary for the c-myc-mediated block in differentiation. The use of a-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC enzyme activity, indicates that ODC is not necessary for the c-myc-mediated differentiation block.

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HEMATOPOIESIS is a profound example of cell homeostasis that is regulated throughout life, whereby a hierarchy of hematopoietic progenitor cells in the bone marrow (BM) proliferate and differentiate along multiple, distinct cell lineages, including the proliferation and differentiation of myeloid precursor cells into mature granulocytes and macrophages. Blocks in differentiation, a major step in tumor progression, can be caused by lesions in genes involved in the control of any one of the interrelated cellular processes comprising the developmental program of terminal differentiation. The differentiation process is controlled by both positive and negative regulators, in which the negative regulators would be expected to be found among genes that control myeloid cell growth. The proto-oncogene, c-myc, which fits this criterion, has been identified in our laboratory to be a negative regulator of terminal myeloid differentiation.

c-myc has been shown to play a pivotal role in growth control, differentiation, and apoptosis, and its abnormal expression has been associated with many naturally occurring neoplasms. c-myc is expressed in almost all proliferating normal cells, and is downregulated in many kinds of cells when they are induced to terminally differentiate. Forced expression of c-myc was shown to block differentiation in several cell types, including myeloid, erythroid, myogenic, preadipocyte, and nerve cells. The c-myc protein contains three structural domains that are homologous to domains found in characterized transcription factors, including a leucine zipper, a helix-loop-helix motif, and an adjacent domain rich in basic amino acids. It is localized to the nucleus, and its binding to DNA is sequence-specific. Recent experimental evidence suggests that c-myc functions as a transcriptional regulator as part of a network of interacting factors. Given the central role c-myc plays in growth control, differentiation, and apoptosis, understanding how c-myc functions will increase our understanding of normal cell development, and how alterations in these processes can lead to malignancy. Toward this end, it would be extremely useful to identify c-myc target genes and to assess their role(s) in the different biologic processes known to be regulated by c-myc.

Recently, evidence has accumulated via several strategies, including the use of the myc transgene, to indicate that the ornithine decarboxylase (ODC) gene is a transcriptional target of c-myc. ODC catalyzes the conversion of ornithine to putrescine, the first and rate-limiting step in polyamine biosynthesis, and has been shown to be crucial to cell growth. Several lines of investigation have shown that ODC activity is critical for cell transformation. It has also been shown that ODC is sufficient to induce cell growth arrest following interleukin-3 withdrawal in IL-3-dependent 32Dc13 myeloid cells, and is a mediator of c-myc--induced accelerated apoptosis in these cells. These data suggest that ODC mediates other c-myc functions in addition to transformation. With this in mind, several lines of experimentation were pursued to ascertain if deregulated ODC expression can negatively regulate myeloid differentiation, and if ODC is a mediator of the differentiation block due to deregulated c-myc expression. The availability of the myeloblastic leukemic M1 cell line that can be induced for terminal differentiation and growth arrest by IL-6 or leukemia inhibitory factor, as well as normal myeloid cells that can be induced to undergo proliferation followed by differentiation and growth arrest, provided an opportunity to study the role of ODC in the myeloid differentiation program.

In this study, it was shown that ODC expression is down-regulated following induction of myeloid differentiation and its associated growth arrest. Furthermore, when induction of differentiation is blocked by deregulated expression of c-myc, ODC continues to be expressed. Using the M1 cell line and M1 variants established in our laboratory, it has been
demonstrated that deregulated expression of ODC has no effect on IL-6-induced terminal differentiation and that ODC is not a mediator of the block in differentiation resulting from enforced expression of c-myc. These data, in conjunction with other findings on the role of ODC and c-myc in transformation and apoptosis, demonstrated that different or overlapping but not identical sets of c-myc target genes are responsible for the effect of c-myc on transformation, apoptosis, and differentiation.

MATERIALS AND METHODS

Cells and cell culture. The differentiation-competent murine M1 myeloid leukemic cell line and M1 cell variants, M1myc and M1myc2, as well as M1neo control cell lines generated at the same time, have been described previously. The data were obtained using the M1myc2 and M1myc2-6 cell lines; however, for each cell variant, three independent cell lines were examined (M1myc2, M1myc5, and M1myc12; and M1myc6-20, and M1myc-55), as well as two control M1neo cell lines, in which the results were similar to the data presented. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY) and 10% horse serum at 37°C in a humidified atmosphere with 10% CO2. Cells were seeded at 0.15 x 10^6/mL with or without appropriate treatments. For RNA extractions at early times after various treatments, cell concentrations were adjusted to produce a final density of greater than 0.25 x 10^6/mL at the time of extraction. Viable cell numbers were determined by trypan blue dye exclusion and counting in a hemocytometer. M1myc2 transfecants were maintained in phenol red–free DMEM-21 (GIBCO) containing 10% dialyzed horse serum, M1 and M1myc2 cells were maintained in the same culture conditions when used as controls. PA317 (American Type Culture Collection, Rockville, MD), a viral packaging cell line, was maintained in DMEM and 10% fetal bovine serum. Brief selection in HAT medium was periodically performed to select for cells retaining the packaging function. Myeloblast-enriched BM cells were isolated from femurs of CD-1 mice (Charles River Laboratories, Wilmington, MA) injected intraperitoneally 3 days earlier with 5 x 10^6 sodium caseinate (DIFCO, Detroit, MI) in phosphate-buffered saline.

Purified human recombinant IL-6 (rIL-6) was a gift from Dr L. Souza (Amen, Inc, Thousand Oaks, CA) and used at a concentration of 100 ng/mL. Recombinant-murine IL-6 (r-mIL-6) was produced by the Omura laboratory at the National Institutes of Health (NIH) and used at a concentration of 100 U/mL. 

The steady-state level of ODC transcripts was reduced following 24 hours of treatment with 100 ng/mL r-mIL-6. ODC transcript levels were high in proliferating M1 cells and were downregulated following induction of terminal myeloid differentiation. To determine if ODC expression is regulated during myeloid differentiation, ODC gene expression was analyzed in both myeloid leukemic M1 cells and myeloid-enriched BM cells following induction for differentiation. ODC transcript levels were high in proliferating M1 cells and were downregulated following the induction of differentiation by IL-6 (Fig 1A). The steady-state level of ODC transcripts was reduced following 24 hours of treatment with IL-6, and was undetectable by 48 hours. Consistent with these results, the enzymatic activity of ODC was reduced to 70% of control levels by 24 hours and decreased to less than 5% of control levels by 3 days. These data show that the regulation of ODC enzyme activity closely parallels the kinetics of ODC mRNA expression during IL-6–induced myeloid differentiation. Following treatment of myeloid-enriched BM with GM-CSF, ODC expression remained high.
in proliferating normal myeloid cells and was downregulated when cells began to undergo terminal differentiation, as seen by Northern blot analysis (Fig 1B) and enzymatic activity (data not shown).

ODC expression also was analyzed following IL-6 treatment of M1myc cells, in which the myeloid differentiation program is blocked at an intermediate stage by deregulated c-myc expression.7 Continuous expression of ODC transcripts was observed up to 3 days following IL-6 simulation of M1myc cells (Fig 1A). The level of ODC enzyme activity detected in M1myc cells following 3 days of IL-6 treatment was only slightly reduced relative to untreated control cells (Fig 1C). These results indicate that deregulated c-myc expression prevents significant ODC downregulation during M1 myeloid differentiation.

**ODC is a c-myc target gene activated by the chimeric mycer transgene.** Recent reports have pointed out that ODC is a downstream target gene of the proto-oncogene, c-myc.24-26 That ODC continues to be expressed in IL-6-treated M1myc cells is consistent with ODC's being a c-myc target gene in differentiating M1 cells. To corroborate this, the M1myc cell lines ectopically expressing the conditional mycer transgene were exploited. Mycer is a chimeric gene, in which the c-myc coding region is juxtaposed to the hormone-binding domain of the estrogen receptor gene, conferring hormone dependence on the function of the c-myc protein. Previously, we have shown that in the absence of estrogen, M1myc cells respond like M1 cells to IL-6; in the presence of estrogen, they respond like M1myc cells. Consistent with these observations, ODC expression was downregulated in M1myc cells treated with IL-6 in the absence of estrogen, and continued to be expressed at elevated levels in the presence of estrogen (Fig 2A).

To establish that ODC is a c-myc target gene, ODC mRNA expression in M1mycer cells was analyzed 32 hours after IL-6 treatment, either with no additional treatment or with estrogen and/or cycloheximide, an inhibitor of protein synthesis, for 3 additional hours. It was observed that ODC expression was induced in the presence of estrogen or estrogen plus cycloheximide (Fig 2B). That ODC mRNA can be induced by the activated chimeric mycer protein in the absence of de novo protein synthesis demonstrates that ODC is a transcriptional target gene of c-myc in differentiating myeloid cells. Similar results were obtained using the estrogen antagonist, hydroxytamoxifen (OHT; data not shown). OHT elicits binding of the estrogen receptor to DNA, but not activation of transcription, eliminating the possibility that ODC is induced by the portion of the estrogen receptor included in the mycer transgene. Consistent with ODC's being a target gene of c-myc, activation of mycer led to an increase in ODC enzymatic activity in IL-6-treated M1mycer cells (Fig 2C).

**Deregulated ODC is not sufficient to block myeloid differentiation.** Altered or deregulated expression of c-myc has been shown to participate in cell transformation, to block terminal differentiation, and to promote apoptosis. ODC, a c-myc target gene, has been shown to be critical for cell transformation26,30 and to be sufficient to induce accelerated apoptosis following IL-3 withdrawal in IL-3-dependent 32Dc13 myeloid cells.30 Taken together with the observations already described, ie, ODC was downregulated following induction of terminal myeloid differentiation and ODC continued to be expressed in M1myc myeloid cells that are blocked for terminal differentiation, it was reasonable to ask if deregulated ODC expression would be sufficient to block myeloid differentiation. To test this hypothesis, M1 cells

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**Fig 1. ODC expression and enzymatic activity following induction of myeloid differentiation.** (A and B) Analysis of M1, M1myc, and BM cells after induction of differentiation by IL-6 (M1 and M1myc) or GM-CSF (BM) at indicated times for expression of ODC, c-myc, and lysozyme, using Northern blot analysis. (C) ODC enzymatic activity at indicated times following induction of differentiation by IL-6. Assays were performed in triplicate, and results are the mean of 3 independent determinations, with standard deviations ≤15%. Each experiment was repeated 3 times, and 1 representative experiment is shown. Blots were stripped and sequentially hybridized with the different probes.
expressing deregulated ODC were established and analyzed for growth arrest and differentiation following treatment with the differentiation inducer, IL-6.

To establish M1 cell lines expressing deregulated ODC, M1 cells were infected with the retroviral expression vector, pMV7-ODC, and individual M1-ODC clones were selected in G418, as described earlier herein. All M1-ODC clones expressed long-terminal-repeat-derived ODC mRNAs (5 kb) in addition to endogenous mRNA (2.4 kb), as determined by Northern blot analysis of total RNA (Fig 3A). In addition, clonally derived M1-ODC cell lines were found to have elevated levels of ODC enzyme activity relative to M1 and M1neo control cells (Fig 3B).

To determine whether deregulated ODC activity was sufficient to block IL-6–induced differentiation, three M1-ODC clones were analyzed for response to IL-6. Following IL-6 treatment, all M1-ODC clones expressed ODC enzyme activity at a level at least as high as that in untreated M1 and M1neo cells (Fig 3B). Therefore, it can be concluded that enforced expression of ODC is not sufficient to block myeloid differentiation program. Interestingly, although ODC is required for entry into the S phase of the cell cycle,39 continued expression of ODC did not prevent IL-6–induced growth arrest (Table 1).

**ODC expression plays no role in the c-myc–mediated block in differentiation.** Although deregulated expression of ODC, one of the c-myc target genes, is not sufficient to block myeloid differentiation, ODC expression may be necessary for the c-myc–mediated differentiation block. This possibility was explored using DFMO, a well-characterized, specific, and irreversible inhibitor of ODC,39 to assess the effect of blocking ODC activity on M1 and M1myc cells uninduced or induced to undergo terminal differentiation.

DFMO is an effective inhibitor of ODC enzyme activity in M1 cells; it rapidly inhibited enzyme activity, with less than 10% of activity remaining after 30 minutes (data not shown). DFMO inhibited ODC enzyme activity in both parental M1 and M1myc cells either treated or untreated with IL-6 (Fig 4A). M1 cells treated concomitantly with IL-6 and DFMO appeared similar to M1 cells treated only with IL-6 with regard to morphology, growth arrest, and induction of Fc and C3 receptors; therefore, DFMO had no deleterious effect on the IL-6–induced developmental program of M1 cells (Fig 4B and Table 2). Since DFMO inhibited ODC enzyme activity of M1myc cells, it was possible to ascertain if ODC activity is required for the c-myc–mediated block in IL-6–induced terminal differentiation. DFMO had no effect on the block in terminal differentiation caused by deregulated expression of c-myc in M1myc cells, demonstrating that ODC expression is not necessary for the c-myc–mediated block in terminal differentiation of M1myc cells (Fig 4B and Table 2).

**DISCUSSION**

In this report, we have shown that ODC is downregulated following induction of myeloid differentiation, and that when differentiation is blocked by deregulated expression of c-myc ODC continues to be expressed. Using M1 cells expressing the conditional mycer chimeric transgene, which
requires estrogen for activation of c-myc function, we demonstrated that induction of ODC transcripts is dependent on estrogen but not on de novo protein synthesis, consistent with the notion that ODC is a c-myc target gene. Establishment of cell lines ectopically expressing ODC has shown that deregulated expression of ODC is not sufficient to block myeloid differentiation; in addition, experiments using the irreversible ODC inhibitor, DFMO, have shown that ODC is not required for the c-myc-mediated block in myeloid differentiation.

Although ODC has been shown to be a c-myc target gene, our data indicate that there are additional regulators of ODC gene expression. These data include the presence of ODC transcripts 24 hours after addition of IL-6 to M1 cells at a
Table 1. Effect of Deregulated ODC Expression on IL-6-Induced Myeloid Differentiation

<table>
<thead>
<tr>
<th>Cell Type (%)</th>
<th>Blast</th>
<th>Inter</th>
<th>Mature</th>
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<tr>
<td><strong>Clone</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
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<td></td>
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<tr>
<td>-</td>
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</tr>
<tr>
<td>+</td>
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<tr>
<td>-</td>
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<td>+</td>
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<td>86</td>
<td>48</td>
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<tr>
<td>M1-ODC3†</td>
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</tr>
<tr>
<td>+</td>
<td>0.35</td>
<td>78</td>
<td>43</td>
</tr>
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</table>

* IL-6 was used at 100 ng/mL. Cells were seeded at a density of 0.15 × 10⁶.
† Determined 3 days after cells were seeded. All values are the mean of 3 independent determinations, with standard deviations ≤15%.
‡ Similar results were obtained with M1-ODC7.

level only slightly diminished compared with levels in untreated cells, although c-myc transcripts and protein are no longer detectable by 18 hours following treatment with IL-6.3,8 Also, in BM c-myc suppression precedes and is more dramatic than ODC suppression. The longer half-life of ODC transcripts relative to c-myc transcripts and protein may account for some of the delay in ODC suppression (data not shown). Conversely, when c-myc continues to be expressed in M1myc cells following treatment with the differentiation inducer, IL-6, ODC has decreased to levels less than those in untreated cells; in other words, IL-6 treatment results in a small decrease in ODC expression despite the presence of c-myc.

It has been shown that ODC is necessary for entry of cells into the S phase of the cell cycle.3,8 Our data demonstrate that ODC expression is not sufficient to allow myeloid cells, which have been stimulated to undergo terminal differentiation and its associated G0/G1 arrest, to continue to cycle. M1-ODC cells treated with the differentiation inducer, IL-6, are growth-arrested, whereas M1myc cells similarly treated continue to cycle.1 Further corroboration of the failure of enforced expression of ODC to block IL-6-induced cell-
Mlmyc cells exhibit a block in IL-6-induced differentiation. Polyamines are unlikely to be present after treatment with cells to undergo GO/G1 arrest. That ODC participates in the inhibitor, DFMO, slows the growth of Mlmyc cells concomitantly with or without ODC, are involved in the failure of M1myc cells to undergo GO/G1 arrest following treatment with IL-6. Intracellular pools of ODC cells to exert a change in response to myeloid differentiation, which included the failure of M1myc cells to undergo GO/G1 arrest following treatment with IL-6 is suggested by the observation that the ODC inhibitor, DFMO, slows the growth of M1myc cells concomitantly treated with IL-6.

The reported effects of ectopic ODC expression on transformation and apoptosis involved elevated levels of ODC, in which the effect on apoptosis in factor-deprived 3D cells was due to ODC levels that were twofold higher than basal levels and the effect on transformation was in the presence of 20 to 100 times the endogenous ODC levels. Therefore, there may be insufficient ODC in M1-ODC cells to exert a change in response to myeloid differentiation inducers. However, it must be kept in mind that M1myc cells exhibit a block in IL-6-induced differentiation and fail to undergo GO/G1 arrest, yet ODC is expressed only at the basal level.

DFMO rapidly inhibits ODC activity, with less than 10% of activity remaining after 30 minutes. As reported, DFMO had no effect on the c-myc-mediated block in differentiation, which included the failure of M1myc cells to arrest in GO/G1 following treatment with IL-6. Intracellular pools of polyamines are unlikely to be present after treatment with DFMO for 3 days, since putrescine and spermidine levels are significantly reduced by 10 and 24 hours, respectively.

It had also been reported that DFMO does not completely arrest cell growth, despite depletion of putrescine and spermidine. This is in agreement with our reported observations that DFMO does not cause growth arrest of M1 and M1myc cells, but causes a reduction in cell number of M1 and M1myc cells, with or without IL-6.

We have previously reported that blocking c-myc expression in both M1 and normal myeloid cells activates or accelerates, respectively, the myeloid differentiation program. Blocking ODC expression by DFMO in proliferating M1 cells has no effect on these cells beyond slowing proliferation; no differentiation-associated characteristics were observed (Table 2).

It is still an open question as to how c-myc exerts effects on differentiation. This study demonstrates that different or overlapping but not identical sets of c-myc target genes are responsible for the effect of c-myc on transformation, apoptosis, and differentiation. Functional analysis of known and unidentified c-myc target genes, separately and in combination, should generate further information toward deciphering how the proto-oncogene c-myc regulates terminal differentiation and its associated growth arrest, as well as transformation and apoptosis.

### Table 2. Effect of Inhibiting ODC Activity on the C-myc-Mediated Block to Myeloid Differentiation

<table>
<thead>
<tr>
<th>Clone</th>
<th>Treatment*</th>
<th>Cell No. (10^6/mL)</th>
<th>Cell Adherence (%) Cell Type (%)</th>
<th>Cell Type (%)</th>
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<tbody>
<tr>
<td></td>
<td>IL-6</td>
<td>DFMO</td>
<td>Blast Inter Med Inter Med Mature</td>
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</tr>
<tr>
<td>M1</td>
<td>-</td>
<td>+</td>
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</tr>
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</table>

* IL-6 was used at 100 ng/mL; DFMO was used at 10 mM/mL. Cells were seeded at a density of 0.15 x 10^6.
† Determined 3 days after cells were seeded. All values are the mean of 3 independent determinations, with standard deviations ±15%.

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The proto-oncogene c-myc blocks myeloid differentiation independently of its target gene ornithine decarboxylase

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