Tiazofurin Induction of Mouse Erythroleukemia Cell Hemoglobin Production in the Absence of Commitment or Changes in Protooncogene Expression

By Matthew L. Sherman, Timothy D. Shafman, Michael S. Colman, and Donald W. Kufe

Tiazofurin (2-β-d-ribofuranosylthiazole-4-carboxamide, NSC 286193), is a synthetic nucleoside inhibitor of inosine monophosphate dehydrogenase and blocks guanine nucleotide biosynthesis. In the present study, we examined the effects of tiazofurin on mouse erythroleukemia (MEL) cell differentiation and protooncogene expression. Tiazofurin induced hemoglobin production in MEL cells in a concentration-dependent manner, as measured by an increase in benzidine staining. Northern blot analysis of MEL cells treated with 7 μmol/L tiazofurin demonstrated accumulation of both α- and β-globin RNA transcripts. This induction of differentiation was blocked by the presence of exogenous guanosine (100 μmol/L). In contrast to the down-regulation of c-myc and c-myb RNA in MEL cells induced by dimethyl sulfoxide (DMSO) or hexamethylene bisacetamide (HMBA), there was no detectable change in levels of these transcripts after tiazofurin treatment. Furthermore, MEL cells induced by tiazofurin did not commit to terminal differentiation. These results suggest a role for guanine nucleotides, at least in part, in the regulation of MEL cell differentiation.

I NOSINE monophosphate (IMP) dehydrogenase activity is responsible for the conversion of IMP to GMP. Levels of this enzyme are elevated in rapidly proliferating cells, whereas low levels are associated with terminal differentiation. An inhibitor of IMP dehydrogenase, tiazofurin (2-β-d-ribofuranosylthiazole-4-carboxamide, NSC 286193), blocks guanine nucleotide synthesis in the human HL-60 promyelocytic leukemia cell line and is a potent inducer of HL-60 cell differentiation. Furthermore, induction of terminal differentiation of HL-60 cells has been associated with a decrease in IMP dehydrogenase activity and depletion of intracellular GTP and GDP.

Friend virus-infected mouse erythroleukemia (MEL) cells have been studied as a model for erythroid differentiation. Certain agents, such as dimethyl sulfoxide (DMSO) and hexamethylene bisacetamide (HMBA), act as inducers of both MEL and HL-60 cells. In contrast, although 12-O-tetradecanoyl-13-acetate (TPA) is a potent inducer of monocytic differentiation in HL-60 cells, it has been reported to be an inhibitor of MEL cell erythroid differentiation. Consequently, the present study was undertaken to determine the effects of tiazofurin on MEL protooncogene expression and appearance of the differentiated MEL phenotype.

MATERIALS AND METHODS

Cell culture. Murine erythroleukemia cells (745-PC-4; provided by Dr D. Housman, Massachusetts Institute of Technology, Cambridge, MA) were maintained as previously described. The MEL cells were grown in suspension culture with varying concentrations of tiazofurin (provided by the National Cancer Institute, Bethesda, MD). Cultures were scored for hemoglobin production (benzidine-positive cells) by the wet benzidine method. Viability was monitored by trypan blue exclusion.

Commitment assay. Commitment to terminal differentiation was assayed in 1.8% methylcellulose as previously described. The colonies were examined 72 hours after being plated. The plating efficiency of uninduced cells was >85%. The degree of commitment was defined, on a percentage basis, as the ratio of benzidine-positive colonies (<64 cells in size) to the number of colonies scored.

Northern blot analysis. MEL cell cytoplasmic RNA was isolated, analyzed, and hybridized as previously described to the following nick-translated 32P-labeled DNA probes: (a) plasmid pBR325 containing a mouse β-major globin DNA; (b) plasmid pBR325 containing a mouse α-globin DNA; (c) pMC-myc-54 containing a 2.2-kilobase (kb) mouse c-myc cDNA insert; (d) pc-myc containing a mouse c-myc cDNA insert (provided by Dr P. Reddy, Hoffman-LaRoche, Nutley, NJ); and (e) pAl plasmid containing a 2.0-kb PstI insert of the chicken β-actin gene. Filters were exposed to x-ray film at −70°C with an intensifying screen.

RESULTS

We monitored the effects of varying concentrations of tiazofurin on MEL cell proliferation. Although treatment with 1 μmol/L tiazofurin had no effect on cell growth, increasing concentrations were associated with decreases in
MEL cell proliferation (Fig 1). Cytostasis was achieved with 10 \( \mu \text{mol/L} \) tiazofurin (Fig 1). We also determined the effects of varying concentrations of tiazofurin on induction of MEL cell differentiation. Tiazofurin at 1 \( \mu \text{mol/L} \) had no detectable effect on the appearance of benzidine-positive cells. However, higher concentrations of this agent induced MEL cell hemoglobin synthesis in a dose-dependent manner (Table 1). Thus, treatment with 10 \( \mu \text{mol/L} \) tiazofurin resulted in maximal induction of benzidine positive cells (39.8%). Cell viability following exposure to 10 \( \mu \text{mol/L} \) tiazofurin remained >95% as determined by trypan blue exclusion, whereas higher concentrations were partially cytotoxic to MEL cells. MEL cells were also treated with tiazofurin in the presence of guanosine to determine if hemoglobin production and loss of proliferation were blocked by repletion of guanine nucleotides. There was no detectable MEL cell hemoglobin production or loss of proliferative capacity induced by tiazofurin in the presence of 100 \( \mu \text{mol/L} \) guanosine (Table 1).

Northern blot analysis of MEL cytoplasmic RNA hybridized to globin DNA probes is shown in Fig 2. Treatment of MEL cells with tiazofurin for various times up to 96 hours resulted in an increase in both \( \alpha \)- and \( \beta \)-globin RNA transcripts (Fig 2A). In addition, this effect was inhibited by the addition of exogenous guanosine to tiazofurin-treated MEL cells (Fig 2B).

We next studied the effects of tiazofurin on MEL cell protooncogene expression. Northern blot analysis of MEL cytoplasmic RNA collected over 96 hours of drug exposure is shown in Fig 3. In contrast to induction of MEL cell differentiation by DMSO or HMBA, treatment with 7 \( \mu \text{mol/L} \) tiazofurin had no detectable effect on levels of \( c\text{-myc} \) or \( c\text{-myb} \) RNA.

Previous studies have demonstrated that certain inducers such as DMSO commit MEL cells to terminal differentiation. Control, DMSO-treated, and tiazofurin-treated MEL cells were grown in methylcellulose to determine the percentage of committed cells. As listed in Table 2, treatment with DMSO for three days before plating in methylcellulose was associated with 65% benzidine-positive colonies. In contrast,

### Table 1. Effect of Tiazofurin on MEL Cell Differentiation

<table>
<thead>
<tr>
<th>Addition</th>
<th>Cell Count (x 10^6/mL)</th>
<th>Benzidine Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.4*</td>
<td>0</td>
</tr>
<tr>
<td>DMSO, 1.8%</td>
<td>5.0*</td>
<td>88.5 ± 1.1</td>
</tr>
<tr>
<td>Tiazofurin (( \mu \text{mol/L} ))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.67*</td>
<td>39.8 ± 0.5</td>
</tr>
<tr>
<td>7</td>
<td>1.3*</td>
<td>35.5 ± 4.7</td>
</tr>
<tr>
<td>5</td>
<td>3.3*</td>
<td>22.2 ± 1.8</td>
</tr>
<tr>
<td>1</td>
<td>4.0*</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0.77†</td>
<td>0</td>
</tr>
<tr>
<td>Tiazofurin 7 ( \mu \text{mol/L} )</td>
<td>0.41†</td>
<td>22.0 ± 0.2</td>
</tr>
<tr>
<td>Guanosine 100 ( \mu \text{mol/L} )</td>
<td>0.97†</td>
<td>0</td>
</tr>
<tr>
<td>Guanosine/tiazofurin</td>
<td>0.78†</td>
<td>0</td>
</tr>
</tbody>
</table>

*MEL cells were seeded at 8 \( \times 10^5/mL \) on day 0. Cell counts and benzidine staining were determined after four days. Concentrations of tiazofurin >10 \( \mu \text{mol/L} \) were cytotoxic.

†Cell counts and benzidine staining were determined after three days of drug treatment.
EFFECTS OF TIAZOFURIN ON MEL CELLS

Fig 3. Effects of tiazofurin on the induction of c-myc and c-myb RNA during MEL cell differentiation. MEL cells were treated with 7 μmol/L tiazofurin for the indicated times. Cytoplasmic RNA was purified as previously described and hybridized to nick-translated probes after transfer to nitrocellulose.

tiazofurin had little if any effect on terminal differentiation of MEL cells.

DISCUSSION

Treatment of MEL cells with agents such as DMSO and HMBA induces a coordinated program of molecular events including expression of globin RNA, heme synthesis, inhibition of growth, and a loss of self-renewal capacity. Other events associated with MEL cell differentiation include changes in c-myc and c-myb gene expression. In contrast, compounds such as hemin fail to induce the entire differentiation program. For example, although hemin increases levels of globin mRNA and hemoglobin, this agent has no effect on growth rates of MEL cells and fails to induce commitment to terminal differentiation.12-15 Furthermore, hemin treatment of MEL cells has no detectable effect on c-myc and c-myb mRNA levels.16

The present studies demonstrate that tiazofurin, like DMSO, HMBA, and hemin, induces MEL cell hemoglobin production as monitored by benzidine staining. Furthermore, tiazofurin increased both α- and β-globin transcripts in these cells. These effects were blocked by addition of exogenous guanosine, suggesting that depletion of guanine nucleotides is related to induction of globin gene expression.

MEL cell differentiation induced by DMSO and HMBA is also associated with a biphasic decrease in the level of c-myc and c-myb transcripts.16-19 Our results indicate that tiazofurin fails to induce changes in c-myc and c-myb expression, despite the inhibitory effects of this agent on MEL cell growth. In this regard, we previously demonstrated that arrest of MEL cell proliferation by ornithine decarboxylase inhibition is not associated with detectable changes in c-myc expression.19 Furthermore, in other cell lines, reduced myb and mvc expression may not be obligatory for phorbol ester-induced growth arrest to occur.20 Our results also demonstrate that tiazofurin has no detectable effect on commitment to terminal MEL cell differentiation. This finding and the absence of changes in c-myc expression are in concert with recent studies on the effects of constitutively expressed c-myc genes transfected into MEL cells that suggest that a decrease in c-myc expression is necessary for commitment to terminal differentiation.21-24 Together, these results suggest that a decrease in c-myc and/or c-myb may be necessary for commitment; however, such decreases are not obligatory for induction of hemoglobin production in MEL cells.

Finally, recent studies have demonstrated that c-myc protein levels are maintained in the presence of declines in c-myc transcripts during erythroid differentiation of MEL cells by DMSO and hypoxanthine.25 These results question the significance of the biphasic decline in c-myc RNA levels in the induction of MEL cell differentiation by DMSO and certain other agents. Furthermore, these studies question whether declines in c-myc protein are necessary for commitment to terminal differentiation. The identification of tiazofurin as a compound which dissociates hemoglobin accumulation from changes in c-myc expression and commitment should provide an opportunity to examine further the molecular mechanisms responsible for induction of the differentiated MEL phenotype.

ACKNOWLEDGMENT

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REFERENCES

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