Effects of Tiazofurin on Protooncogene Expression during HL-60 Cell Differentiation

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ABSTRACT

The synthetic nucleoside analogue, tiazofurin (2-β-D-ribofuranosylthiazole-4-carboxamide, NSC 286193) is an inhibitor of the enzyme inosine monophosphate (IMP) dehydrogenase and depletes guanine nucleotide pools. In the present study, we have monitored the effects of tiazofurin on human HL-60 promyelocytic cell differentiation and protooncogene expression. Tiazofurin (10 μM) induced a more differentiated HL-60 cell phenotype as determined by histochemical staining and decreased myeloperoxidase gene expression. This induction of differentiation was associated with a loss of proliferative capacity and decreases in clonogenic survival. The results also demonstrate that tiazofurin induces a down-regulation of c-myc mRNA levels. In contrast, there was no detectable change in the level of 3.8-kilobase c-myc transcripts. Furthermore, treatment of HL-60 cells with tiazofurin resulted in the appearance of an additional c-myc mRNA with an apparent size of 3.3 kilobases. The addition of guanosine to tiazofurin-treated HL-60 cells prevented the down-regulation of c-myc transcripts and also inhibited induction of the 3.3-kilobase c-myc transcript. Moreover, this additional transcript was not detected during induction of HL-60 cells by dimethyl sulfoxide, tumor necrosis factor, and retinyl acetate, but was induced by another IMP dehydrogenase inhibitor, mycophenolic acid. These results suggest a role for guanosine ribonucleotides in the regulation of c-myc and c-myc gene expression during HL-60 cell differentiation. The results also suggest that changes in c-myc expression can be dissociated from that of c-myc and induction of myeloid differentiation.

INTRODUCTION

IMP3 dehydrogenase catalyzes the conversion of IMP to xanthine monophosphate, a step required for the de novo synthesis of guanine nucleotides. High levels of this enzyme have been found in rapidly proliferating cells while low levels have been associated with terminal differentiation (1). Induction of terminal differentiation of the human HL-60 promyelocytic leukemia cell line with dimethylformamide, retinoic acid, and hypoxanthine has been associated with a decrease in IMP dehydrogenase activity and depletion of intracellular guanine nucleotides (2). Moreover, tiazofurin, (2-β-D-ribofuranosylthiazole-4-carboxamide, NSC 286193), a specific inhibitor of IMP dehydrogenase, decreases HL-60 cell guanylate synthesis from inosine monophosphate, depletes intracellular guanine nucleotide pools, and induces a differentiated phenotype (3). Other inhibitors of IMP dehydrogenase, such as selanazofurin, mycophenolic acid, and 3-deazaguanosine, are also potent inducers of HL-60 cell differentiation (2, 4). Furthermore, tiazofurin has recently been shown to reduce leukemic cell GTP levels and induce maturation of blasts in a patient with refractory acute myelogenous leukemia (5). These findings have suggested that guanine nucleotides play a role in the regulation of myeloid cell differentiation.

HL-60 cells undergo myeloid differentiation when exposed to inducers such as DMSO, hexamethylene bisacetamide, or retinoic acid. These cells can also be induced along a monocytic pathway by treatment with phorbol esters or 1,25-dihydroxyvitamin D3. Previous studies have shown that HL-60 cell differentiation is associated with specific changes in protooncogene expression. For example, the induction of HL-60 differentiation to either granulocytes or monocytes is associated with a loss of proliferative capacity and a decrease in c-myc expression (6–9). Furthermore, myeloid differentiation has also been associated with a decrease in the level of c-myc transcripts (10, 11). In contrast, while N-ras and c-raf expression are not altered during HL-60 differentiation (9, 12), the levels of c-fos, c-fms, and c-sis RNA are increased when these cells are induced along the monocytic lineage (13, 14).

The effects of tiazofurin on protooncogene expression in HL-60 cells have not been examined previously. In the present study, we demonstrate that tiazofurin down-regulates c-myc expression. However, in contrast to other inducers of HL-60 cell differentiation, tiazofurin failed to decrease levels of the 3.8-kilobase c-myc transcript and even induced the appearance of a 3.3-kilobase c-myc-related mRNA. Similar effects were observed with other inhibitors of IMP dehydrogenase.

MATERIALS AND METHODS

Cell Culture. The HL-60 promyelocytic cells were grown in RPMI 1640 media containing 15% fetal bovine serum supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1 mM nonessential amino acids. Tiazofurin (provided by the National Cancer Institute, Bethesda, MD) was diluted in media without serum or in PBS and added directly to the cell cultures. DMSO (Fisher), retinal (Sigma), TNF (Asahi Chemical Industry), and mycophenolic acid (Sigma) were used at a final concentration of 1.25%, 1 μM, 100 U/ml, and 10 μM, respectively. Cyto centrifuge smears of cultured cells were examined for NSE staining and NBT reduction (15, 16). The percentage of positive cells was determined by counting 200 cells in duplicate. Viability was determined by trypan blue exclusion.

Clonogenic Survival. HL-60 cells were exposed to 10 μM tiazofurin for varying times up to 72 h. After three washes with ice-cold PBS, the cells (1 × 103/35-mm dish) were grown in culture media and 0.3% agarose (Bacto-Agar; Difco Laboratories, Detroit, MI) on a base layer of 0.5% agarose. The cultures were incubated and colonies greater than 50 cells were counted after 7 days.

Preparation of RNA and Hybridization. Total cellular RNA was isolated by the guanidine isothiocyanate-cesium chloride technique as described previously (17). Total cellular RNA (20 μg) was subjected to electrophoresis in a 1% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose paper, and hybridized to one of the following 32P-labeled DNA probes: (a) the 1.5-kilobase Cldl/EcoRI fragment containing the human c-myc 3' exon purified from the pmC41-3 RC plasmid (18); (b) the 2.0-kilobase EcoRI fragment of the human c-myc gene purified from the F8 (c-myc) plasmid (19); (c) the 2.3-kilobase XhoI/NcoI fragment of the c-fos gene purified from the pc-fos(human)-1 plasmid (20); (d) the 1.0-kilobase PstI fragment of the v-fms gene purified from...
the pSM3 plasmid (21); (e) the pMPO2 plasmid containing a 2.2-
kilobase insert of the human myeloperoxidase gene (22); (f) the pA1
plasmid containing a 2.0-kilobase Psrl insert of the chicken β-actin
gene (23); and (g) the pc-myb plasmid containing a mouse c-myb cDNA
insert (provided by Dr. E. P. Reddy, Hoffman-LaRoche). The hybridiz-
ations were carried out for 16–24 h at 42°C in 50% (v/v) formamide,
2x SSC (SSC: 0.15 M sodium chloride, 0.015 M sodium citrate), 1x
Denhardt’s solution, 0.1% (w/v) sodium dodecyl sulfate and 200 µg/
ml salmon sperm DNA. Filters were washed and exposed to Kodak X-
Omat XAR film using an intensifying screen. For some experiments,
total cellular RNA was enriched for poly(A) mRNA using oligo(dT)-
cellulose (Collaborative Research, Inc., Lexington, MA) as described
(24).

RESULTS

Previous studies have demonstrated that inhibitors of IMP
dehydrogenase deplete guanine nucleotide pools in HL-60 cells
and increase functional characteristics, such as NBT reduction
and phagocytosis (2, 3). In the present studies, tiazofurin was
similarly used to induce HL-60 cell maturation. This agent
increased both NBT reduction and NSE staining of HL-60 cells
in a dose- and time-dependent manner. Maximal induction of
these characteristics was obtained with 10 µM tiazofurin at 5
days of treatment, and these effects were associated with a
decrease in cell proliferation without evidence of cytotoxicity
(data not shown). In addition, clonogenic survival of HL-60
cells treated with 10 µM tiazofurin was progressively decreased
at 24, 48, and 72 h of exposure (Table 1). These findings thus
confirmed that tiazofurin induces terminal maturation of HL-
60 cells. Moreover, the effects of tiazofurin on both growth
and maturation were reversed by the addition of 100 µM guanosine
to bypass inhibition of guanylate synthesis from IMP (Table 2).

We next studied the effects of tiazofurin-induced HL-60 cell
differentiation on protooncogene expression. Northern blot
analysis of HL-60 cellular RNA collected over 48 h of drug
exposure is shown in Fig. 1. Treatment with 10 µM tiazofurin
resulted in a progressive decline in levels of the 2.4-kilobase
c-myc transcript. In contrast, there was no detectable change in
levels of the 3.8-kilobase c-myb mRNA. These findings were
also associated with the absence of a detectable effect of tiazo-
furin on actin gene expression. Furthermore, recent studies have demonstrated that myeloid differentiation is associated
with decreased expression of the MPO2 gene (22). Similar

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a HL-60 cells were treated with 10 µM tiazofurin for the indicated times. Control or untreated cultures resulted in 423 ± 39 colonies. The results are expressed as mean ± SD (n = 2).

Table 2 Effect of guanosine on tiazofurin-treated HL-60 cells

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Fig. 1. Effects of tiazofurin on c-myc, c-myb, actin, and MPO2 gene expression
in HL-60 cells. HL-60 cells were treated with 10 µM tiazofurin for the indicated
levels of c-myc and c-myb transcripts were monitored in tiazo-
the induction of the 3.0-kilobase c-myc transcript (Fig. 2B).
Finally, while c-fos and c-fms mRNAs are induced during
degree of HL-60 cell differentiation was maximal at 5 days. Consequently,
levels of c-fos and c-fms transcripts were undetectable in the tiazofurin-
treated cells. When using stringent washing conditions (45°C,
0.1x SSC). Similar results were obtained when using poly(A)-
enriched mRNA or after hybridizing with a c-myb cDNA probe
(data not shown). Furthermore, the addition of 100 µM guano-
sine inhibited both the decrease in c-myc expression, as well as
the induction of the 3.3-kilobase c-myc transcript (Fig. 2B).
levels by 3 and 5 days of drug exposure (Fig. 2A). Moreover,
although there was no detectable effect of tiazofurin on levels
of the 3.8-kilobase c-myb transcript with longer exposures, a
smaller mRNA (approximately 3.3 kilobases) which hybridized
to the c-myc probe was clearly induced by 3 days (Fig. 2A). The
3.3-kilobase c-myc transcript was detectable in the tiazofurin-
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the induction of the 3.3-kilobase c-myc transcript (Fig. 2B).
Finally, while c-fos and c-fms mRNAs are induced during
monocytic differentiation of HL-60 cells (13, 14), these tran-
scripts were undetectable in the tiazofurin-treated cells.

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TIAZOFURIN AND HL-60 CELL DIFFERENTIATION

DISCUSSION

The present studies confirm previous reports (2–4) that inhibition of IMP dehydrogenase induces maturation of HL-60 cells. Thus, tiazofurin increased NSE staining and NBT reduction by these cells in a concentration- and time-dependent manner. Although the precise mechanism responsible for this induction of HL-60 differentiation is unclear, other work has indicated that depletion of GTP pools results in disruption of glycogen metabolism (27). Furthermore, recent work has demonstrated that myeloid differentiation is associated with decreases in myeloperoxidase gene expression (22) and similar results were obtained in the tiazofurin-treated HL-60 cells. We also found that tiazofurin decreased levels of c-myc mRNA. The decrease in c-myc expression induced by tiazofurin is in concert with that observed after treatment of HL-60 cells with a variety of agents that induce differentiation along the granulocytic or monocytic lineages (6–9). Other studies have demonstrated that decreased c-myc expression in differentiated HL-60 cells is due to down-regulation of transcription by a block in elongation (28); however, the present work has not determined whether a similar mechanism exists in tiazofurin-treated cells. The decrease in c-myc transcripts by tiazofurin may be related to tiazofurin-induced inhibition of HL-60 growth, since the c-myc protein has been shown to be functionally involved in cellular growth (29).

The human c-myc gene is almost exclusively expressed in malignant hematopoietic cell lines and in cells from primary hematopoietic malignancies (30). High levels of c-myc mRNA have been previously found in HL-60 cells, while these transcripts become undetectable following induction of differentiation (10). In the present studies, tiazofurin had little, if any, effect on levels of the 3.8-kilobase c-myc transcript, despite induction of a more differentiated phenotype and down-regulation of the c-myc and MPO2 genes. Moreover, tiazofurin treatment resulted in the appearance of a smaller transcript which hybridized to the c-myc probe. This smaller transcript became detectable after 48 h of tiazofurin treatment and was similarly induced by mycophenolic acid, another inhibitor of IMP dehydrogenase. The induction of an additional c-myc transcript may be related to the recent finding that alternative internal splicing of c-myc mRNA occurs in both normal and tumor cells (31). Other studies have suggested that splicing errors induced by viral integration into the c-myc locus may play a crucial role in the activation of this gene (32). The functional significance of the additional c-myc transcript in tiazofurin-treated HL-60 cells is unclear. Furthermore, the mechanism responsible for the additional c-myc transcript is unknown, although the data would suggest that depletion of guanine nucleotides is related to the finding.

The present studies thus confirm that inhibitors of IMP dehydrogenase induce HL-60 cell differentiation, but in contrast to other inducers of these cells (i.e., DMSO, TNF, and retinoic acid), tiazofurin, and mycophenolic acid have distinct effects on c-myc expression. Previous studies in HL-60 cells have indicated that expression of both c-myc and c-myb is linked to DNA replication (33). However, the present findings that c-myc transcripts remain detectable despite tiazofurin-induced growth inhibition would suggest that more than one mechanism is responsible for regulation of this gene. In this regard, c-myc mRNA levels appear to vary with cellular proliferation as a result of posttranscriptional mechanisms, while high levels of c-myc transcripts have also been found in quiescent thymocytes as a result of increased c-myc transcription.

c-mycphenoic acid, was also associated with induction of the additional c-myb transcript (Fig. 3B). Thus, induction of HL-60 cell differentiation with inhibitors of IMP dehydrogenase is associated with distinct effects of c-myb gene expression.
(34). Although the precise mechanisms responsible for the regulation of c-myc and c-myb expression during HL-60 cell differentiation are unknown, the present results demonstrate that the down-regulation of c-myc transcripts can be dissociated from that of c-myb and from induction of myeloid differentiation. Other studies have demonstrated an absence of declines in c-myb mRNA levels following phorbol ester treatment and growth arrest of murine myelomonocytic leukemic cells with an abnormal c-myb locus (35). Lastly, a c-myb expression can also be attenuated by a block in transcriptional elongation (36).

The different mechanisms utilized for regulating expression of c-myb and other genes may be dependent upon extracellular signals. G proteins are known to regulate receptor-mediated responses to extracellular hormones and transduce signals via membrane bound enzymes, such as adenylyl cyclase and phospholipase C. The depletion of GTP pools by IMP dehydrogenase inhibitors could conceivably interfere with G protein function. In this regard, it would be of interest to determine whether depletion of guanine nucleotides by tiazofurin alters G protein-mediated signal transduction events that involve, for example, regulation of adenylyl cyclase activity.

REFERENCES

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