Inhibition of N-myc Expression and Induction of Apoptosis by Iron Chelation in Human Neuroblastoma Cells

Liju Fan, Jaya Iyer, Shaoxian Zhu, Kevin K. Frick, Randall K. Wada, Allen E. Eskensazi, Patricia E. Berg, Naohiko Ikegaki, Roger H. Kennett, and Christopher N. Frantz

Department of Pediatrics and the Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland 21201 [L. F., A. E. E.]; Department of Pediatrics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 [J. I., K. K. F.]; Pediatric Oncology Branch, National Cancer Institute, NIH, Gaithersburg, Maryland 20892 [S. Z.]; Molecular Carcinogenesis, Cancer Research Center of Hawaii and the Kapi'olani Health Research Institute, Honolulu, Hawaii 96813 [R. K. W.]; Department of Biochemistry and Molecular Biology, The George Washington University, Washington, DC 20037 [P. E. B.]; Division of Oncology, The Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104 [N. I.]; Department of Biology, Wheaton College, Wheaton, Illinois 60187 [R. H. K.]; and Department of Medicine, Children’s Hospital and Department of Pediatric Oncology, Dana Farber Cancer Institute, Boston, Massachusetts 02115 [C. N. F.]

ABSTRACT

Neuroblastoma is the second most common solid malignancy of childhood. Overexpression of the amplified N-myc gene in the tumor cells may be associated with poor patient prognosis and may contribute to tumor development and progression. The use of deferoxamine mesylate (DFO), an iron chelator, to treat neuroblastoma is being investigated in national clinical studies. We show here by TUNEL assay and DNA ladder that DFO induces apoptosis in cultured human neuroblastoma cells, which is preceded by a decrease in the expression of N-myc and the altered expression of some other oncopgenes (up-regulating c-fos and down-regulating c-myb) but not housekeeping genes. The decrease in N-myc expression is iron-specific but does not result from inhibition of ribonucleotide reductase, because specific inhibition of this iron-containing enzyme by hydroxyurea does not affect N-myc protein levels. Nuclear run-on and transient reporter gene expression experiments show that the decrease in N-myc expression occurs at the level of initiation of transcription and by inhibiting N-myc promoter activity. Comparison across neuroblastoma cell lines of the amount of residual cellular N-myc protein with the extent of apoptosis measured as pan-caspase activity after 48 h of iron chelation reveals no correlation, suggesting that the decrease in N-myc expression is unlikely to directly mediate apoptosis. In conclusion, chelation of cellular iron by DFO may alter the expression of multiple genes affecting the malignant phenotype by multiple pathways. Given the clinical importance of N-myc overexpression in neuroblastoma malignancy, decreasing N-myc expression by DFO might be useful as an adjunct to current therapy.

INTRODUCTION

Neuroblastoma is the second most common solid tumor in children. It is a malignant neoplasm of childhood that arises in the adrenal medulla or sympathetic ganglia. Survival of children with neuroblastoma correlates with the clinical stage of disease, specific histological features, and amplification (increased copy number) of the N-myc oncogene in the tumor cells (1, 2). Some have found a correlation between a poor outcome of neuroblastoma and a high degree of tumor N-myc expression in children > 1 year of age at diagnosis (3, 4). The N-myc gene is not expressed in most normal adult tissues, but it is highly expressed in embryonal and fetal neuronal tissues (5). The N-myc protein is a transcription factor that heterodimerizes with the Max protein. This dimer binds, in a sequence-specific fashion, to the promoters of certain key cellular genes (6). The enhanced expression of N-myc results in changes in neuroblastoma cell phenotype that contribute to tumor development and progression, including metastasis and invasiveness (6, 7).

Iron may play an unusually important role in the viability and proliferation of neuroblastoma. Iron is required by nearly all organisms, and it facilitates critical cellular biochemical processes including electron transfer and respiration reactions in mitochondria, conversion of ribonucleotides to deoxyribonucleotides by RR3 in DNA synthesis, O2 transfer by hemoglobin, and the activities of many other metalloenzymes. Inhibition of RR has been proposed as the cause of the growth arrest that occurs in cells deprived of iron (8–12), because this enzyme requires iron to reduce ribonucleotides to form the substrates for DNA synthesis. The regulated uptake and availability of iron is closely tied to cellular proliferation. Three proteins, the iron-transport protein transferrin, the transferrin receptor on the cell surface, and ferritin, the intracellular iron storage protein, are essential for making iron available for cellular use and for detoxifying excess iron. Many neuroblastomas produce very large amounts of ferritin (13). In addition to being incorporated into heme proteins and enzymes, iron may participate more directly in the regulation of cell growth and apoptosis of neuroblastoma. Neuroblastoma cells appear to be unusually sensitive to the cytotoxic and cytostatic effects of iron chelators (14). The use of DFO, a compound that chelates iron, to treat neuroblastoma in children is being investigated in national clinical studies.

In light of the clinical importance of N-myc overexpression in neuroblastoma malignancy, we report here a correlation between DFO-induced apoptosis and decreased N-myc expression in neuroblastoma cell lines with an amplified N-myc oncogene. Different caspase activity in these cell lines does not seem to result directly from a similar decrease in N-myc cellular protein, suggesting that the decrease in N-myc expression is unlikely to directly mediate apoptosis. Remarkably, the DFO-induced decrease in N-myc protein is not mediated through inhibition of RR. We also show that iron chelation by DFO, although decreasing N-myc transcription, alters transcription of some other oncogenes, including c-fos and c-myb, but not housekeeping genes. The specific inhibitory effects of iron chelation by DFO were characterized further on N-myc transcription initiation and on the transient expression of N-myc promoter activity.

MATERIALS AND METHODS

Reagents. DFO and all other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO). A stock solution of DFO was prepared in water, sterile filtered, and stored in aliquots at −20°C.

Cells and Cell Culture. The LA-N-1 and LA-N-5 neuroblastoma cell lines were originally obtained from Dr. Robert C. Seeger, University of California at Los Angeles School of Medicine, Los Angeles, CA. The IMR-32 neuroblastoma cell line was purchased from American Type Culture Collection.

3 The abbreviations used are: RR, ribonucleotide reductase; DFO, deferoxamine mesylate; FBS, fetal bovine serum; CAT, chloramphenicol acetyltransferase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Received 5/24/99; accepted 11/20/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 We acknowledge the support of the American Institute for Cancer Research (to L. F.), the Children’s Cancer Foundation (to C. N. F.), and NIH Grant NS34432 (to R. K. W.).

2 To whom requests for reprints should be addressed, at Department of Pediatrics, University of Maryland School of Medicine, Room 10-044A, BB1, 655 W. Baltimore Street, Baltimore, MD 21201.
purified after incubation of the nuclei with [32P]UTP. The targeted sequences, exon 1, exon 2, or exon 3 of N-myc as designated, were cloned into M13 mp 10 or mp 11 in each orientation to yield single-stranded targets for detection of either sense or antisense transcription (a gift of Geoffrey Krystal, Medical College of Virginia, Richmond, VA; Ref. 17). Double-stranded probes for human β-actin were used to normalize for extent of hybridization. Single-stranded targets and double stranded targets were dotted onto nitrocellulose using a Bio-Rad filter manifold and baked at 80°C for 2 h under vacuum. Prehybridization and hybridization were carried out as described (18, 19).

**RESULTS**

We studied the concentration of DFO required to decrease cell number by 50% (IC50) over 5 days in a variety of cell lines. All cell lines were cultured in the same medium and serum concentration to avoid having different amounts of iron available in the medium. In 11 different human neuroblastoma cell lines tested, the IC50 ranged from 0.6 to 18.7 μM with a median of 1.8 μM DFO. The most sensitive cell lines demonstrated extensive blebbing with massive cell death after 24 h of exposure to DFO, suggesting extensive apoptosis. In contrast, in eight non-neuroblastoma human solid tumor cell lines tested, the IC50 ranged from 1.5 to 10.2 μM with a median of 4.0 μM DFO, and rapid extensive cell death was not apparent. For additional studies, we chose LA-N-1, an average human neuroblastoma cell line with an IC50 of 1.8 μM DFO.

**DFO Induces Apoptosis in Cultured Human Neuroblastoma Cells.** TUNEL assay was performed on LA-N-1 cells cultured with or without 50 μM DFO as described in “Materials and Methods.” The time course of appearance of apoptotic cells is shown in Fig. 1A. Few apoptotic cells were observed 24 h after DFO exposure. The number of apoptotic cells/culture started to increase after 48 h of DFO exposure. It continued to rise progressively until eventually >80% of cells were apoptotic at 96 h. In Fig. 1B, laddering of cellular genomic DNA from LA-N-1 cells treated with 50 μM DFO for 96 h confirmed that DFO induces apoptosis when compared with the control. In addition, the total number of cells/culture decreased between 24 and 48 h of DFO (not shown), confirming the cytotoxic effects of DFO on neuroblastoma cells. In apoptosis, caspases are responsible for proteolytic cleavages that lead to cell disassembly and are involved in upstream regulatory events. Pan-caspase activity assay was performed on IMR-32, LA-N-1, LA-N-5, and KAG cells cultured in the presence of 50 μM DFO with or without 50 μM FeSO4 for 48 h as described in “Materials and Methods.” The percentage of cells with pan-caspase activity was significantly higher in DFO-treated cultures of LA-N-1, LA-N-5, or IMR-32, respectively, than in that of the controls. Although no pan-caspase activity was detectable in either treated or control cultures of KAG at 48 h (Fig. 1C), a significant increase in the
percentage of pan-caspase positive cells (40%) was observed after DFO treatment for 96 h (6.6% ± 0.3%) when compared with the control (4.7% ± 0.4%; P < 0.005). These consistent and reproducible results suggest that, albeit with different degrees of sensitivity, DFO induces apoptosis in multiple neuroblastoma cell lines.

Fig. 1. DFO induces apoptosis in cultured human neuroblastoma cells. A, time course of DNA fragmentation by TUNEL assay. One million cells cultured in RPMI 1640 + 3% FBS were exposed to 50 μM DFO or water for 24, 48, 72, and 96 h. Floating and attached cells were pooled and fixed. Each value represents the mean of replicate determinations from one of two independent experiments. A minimum of 500 cells was scored for the incidence of apoptosis in each determination. ■, control with water; □, with 50 μM DFO. Bar, SE. B, DNA fragmentation by DNA laddering. Cells were cultured with 50 μM DFO or water in RPMI 1640 + 3% FBS for 96 h. DNA was isolated separately from three cultures and dissolved in 10 μM Tris (pH 8.0) and 1 μM EDTA (pH 8.0) buffer. DNA (∼10 μg) was resolved by electrophoresis in 1% agarose gel and stained in ethidium bromide. Lanes 1–5, controls with water; Lanes 6–8, treated with 50 μM DFO for 96 h; Lane M, 100-bp ladder. C, pan-caspase activity by flow cytometry. One million cells were cultured in the presence of 50 μM DFO with or without 50 μM FeSO4 in RPMI 1640 + 3% FBS for 48 h. Pooled, floating, and attached cells were counted and incubated with the fluorescein-labeled inhibitor at 37°C for 1 h. Ten thousand cells were analyzed by single-color flow cytometry on the FL1 channel and the percentage of cells with pan-caspase activity was determined. Each value represents the mean of replicate determinations from one of two independent experiments. Gating was used for LA-N-5 when clumping occurred. □, control with DFO and FeSO4; ■, with 50 μM DFO only. Bar, SE; *, Student’s t test, one-tailed, P = 0.005; **, P < 0.0025.

Fig. 2. Effect of DFO and hydroxyurea on cellular N-myc protein. Cells were cultured in RPMI 1640 + 10% FBS. Western blotting was performed loading equal amounts of protein/lane as described in “Materials and Methods.” The same experiment was repeated more than twice. A, time course. Cultures were exposed to 50 μM DFO or water for the indicated time intervals. B, dose response. Cultures were exposed to the indicated concentrations of DFO for 24 h. C, cultures were exposed for 30 h with no addition (control), with 50 μM DFO alone, or with 50 μM DFO and 50 μM FeSO4, ZnSO4, CuSO4, CaCl2, or MgCl2, as designated. The salts without DFO had no effect on N-myc (data not shown). D, cultures were exposed for 48 h to the indicated concentrations of hydroxyurea (in mM) or aphidicolin (in μg/ml).

DFO Decreases Cellular Expression of N-myc Protein. The decrease in cellular N-myc protein content over time in LA-N-1 cells cultured with or without 50 μM DFO was determined by Western blot and is shown in Fig. 2A. The cellular N-myc protein level started to decrease by 12 h of exposure to 50 μM DFO and continued to decrease for 2 days until it was barely detectable. In cells cultured without DFO, the level of N-myc expression remained approximately constant, although a slight increase was seen after 3 days in some experiments. Increasing DFO concentration resulted in proportionate decreases in cellular N-myc protein in cells cultured for 24 h (Fig. 2B). Other compounds that chelate iron well, but not those that chelate iron weakly (EDTA and diethylenetriaminepentaacetic acid), also decreased N-myc in a time- and concentration-dependent manner.4 The addition of ferrous sulfate to the medium at an equimolar concentration prevented the decrease in N-myc induced by 50 μM DFO, but the addition of equimolar Zn2+, Cu2+, or Mg2+ did not (Fig. 2C), suggesting that chelation of Fe2+ is specifically involved in the decreased N-myc expression.

Hydroxyurea and Aphidicolin Do Not Decrease Cellular N-myc Protein. DFO and other iron chelators are known to cause cell growth arrest and to inhibit RR (8–12). To determine whether the DFO-induced decrease in N-myc expression was mediated by RR, LA-N-1 cells were incubated with hydroxyurea, another inhibitor of the enzyme (20). Hydroxyurea treatment resulted in no change in cellular N-myc protein (Fig. 2D) but caused complete growth arrest (data not shown). Therefore, the decrease in N-myc caused by iron deprivation is not mediated by inhibition of RR. Another possible mechanism of the regulation of N-myc by iron is by growth arrest. Like hydroxyurea, aphidicolin induces growth arrest of cells, in this case by inhibition of DNA polymerase α (21). The addition of aphidicolin to LA-N-1 cells completely inhibited cell growth (data not shown) but had no effect on N-myc levels (Fig. 2D). Similarly, the arrest of neuroblastoma cell

4 C. N. Frantz, unpublished data.
growth caused by deprivation of an amino acid, isoleucine (22), does not affect N-myc expression (data not shown). Thus, the mechanism of N-myc regulation by iron chelation is not simply attributable to the arrest of cell growth or the decrease in RR activity.

**DFO Decreases N-myc Cellular Protein in Multiple Neuroblastoma Cell Lines.** We studied N-myc expression by Western blot in five other neuroblastoma cell lines with amplified N-myc and found that there is a common sensitivity to DFO among them. In all these neuroblastoma cell lines, LA-N-5, IMR-32, KAG, KANR, and NGP, N-myc protein levels decreased after 24 h exposure to 50 μM DFO when compared with the control, although the degree of inhibition varied among the cell lines (Fig. 3). The amount of residual cellular protein was similar in IMR-32 and LA-N-5, although much higher pan-caspase activity was induced in IMR-32 than in LA-N-5 by the same concentration of DFO.

**DFO Specifically Inhibits Transcription of N-myc and Other Proto-oncogenes But Not Housekeeping Genes.** We examined the molecular basis of the effects of iron chelation on N-myc transcription. Northern blot analysis revealed that incubation of LA-N-1 cells with 50 μM DFO induced a progressive decrease in N-myc mRNA compared with control cultures (Fig. 4A) without affecting the mRNA level of the housekeeping gene, GAPDH. The decrease was ∼6-fold when measured by densitometry after 24 h of incubation with DFO (not shown), and the rate of decrease was similar to that seen with N-myc protein (Fig. 2A). Northern blot also showed that iron chelation resulted in an increase in transferrin receptor mRNA (Fig. 4A), demonstrating that DFO was effectively decreasing the intracellular iron available to regulate biochemical processes in the cells (23). We also examined cellular mRNA levels of c-myb, c-fos, and c-jun. Although no change was seen in c-myb and c-jun, DFO induced a marked increase in c-fos (Fig. 4B). ∼7-fold as measured by densitometry (not shown). These changes were prevented by coinubation of cells with a concentration of ferrous sulfate equimolar to that of DFO (Fig. 4B). The increase in c-fos and the lack of change in c-jun contrasts with the decrease in N-myc mRNA, demonstrating the specificity of the changes. Similar changes in proto-oncogene mRNA levels were seen in three other N-myc-amplified human neuroblastoma cell lines incubated with DFO, except that c-myb mRNA decreased in one cell line (data not shown).

**Effects of DFO on Stability of N-myc mRNA and Protein.** Cycloheximide at 20 μM and actinomycin D at 5 μg/ml, respectively and effectively, blocked protein and RNA synthesis in LA-N-1 cells.4 Cycloheximide at 20 μM (24) was added to LA-N-1 cells after incubation for 24 h with and without DFO. At timed intervals, cellular N-myc protein was measured by Western blot and densitometry. The half-life of the N-myc protein was 62.9 min with and 65 min without 50 μM DFO (Table 1). The concentration of the N-myc mRNA was measured similarly at timed intervals after the addition of actinomycin D at 5 μg/ml (25). The half-life of the N-myc mRNA was found to be 57 min with or 59 min without 50 μM DFO (Table 1). The half-life for N-myc protein in the control was in the same range, though four times longer for N-myc mRNA than those reported in other neuroblastoma cell lines (24, 25). Thus, iron chelation has little effect on the stability of N-myc protein or on mRNA in LA-N-1 cells.

**DFO Inhibits the Initiation but Not the Elongation of N-myc Transcription.** To determine whether the initiation or elongation of N-myc transcription was affected by DFO, a nuclear run-on assay was performed comparing LA-N-1 cells that had been incubated with and without 50 μM DFO for 24 h. Transcripts hybridizing to a probe from each of the three N-myc exons were examined. Elongation of c-myc and l-myc transcripts may be delayed, especially at points in the first exon (26, 27). Iron chelation with DFO resulted in marked decreases in transcripts binding to all three exons (Fig. 5). Hybridization to each exon was reduced to a similar extent (Fig. 5). Therefore, incubation with DFO decreased initiation of N-myc transcription rather than

---

**Table 1:** Effect of DFO on stability of N-myc protein and mRNA

<table>
<thead>
<tr>
<th></th>
<th>+DFO</th>
<th>−DFO</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-myc protein</td>
<td>62.9</td>
<td>64.9</td>
</tr>
<tr>
<td>N-myc mRNA</td>
<td>57.0</td>
<td>59.0</td>
</tr>
</tbody>
</table>

---

**Fig. 3.** Effect of DFO on cellular N-myc protein in neuroblastoma cell lines. Five human neuroblastoma cell lines were cultured in RPMI 1640 + 10% FBS. Western blotting was performed as described in Fig. 2. Cultures were exposed to 50 μM DFO or water for 24 h. The same experiment was repeated more than three times. Minus, control; plus, 50 μM DFO.

**Fig. 4.** Effect of DFO on N-myc mRNA levels. A, time course. Cultures were exposed to 50 μM DFO or water and harvested at the timed intervals designated (in days). Cellular mRNA was prepared, electrophoresed, and blotted on Nytran as described by the manufacturer. B, cultures were exposed for 24 h to no addition (control), 50 μM DFO, or 50 μM DFO with 50 μM FeSO₄. Northern blots were hybridized with N-myc and GAPDH as above, or with c-fos (0.6-kb fragment of exon 4 obtained by PCR), c-myb (EcoRI-EcoRI cDNA fragment), or c-jun (1.9-kb cDNA fragment).

---

Downloaded from cancerres.aacrjournals.org on July 21, 2017. © 2001 American Association for Cancer Research.
expression of an N-myc promoter (\(-\text{myc}\) promoter activity, we investigated the transient initiation of transcription.

Transcripts are unlikely to play a role in the changes in N-myc expression. Therefore, antisense transcription (Fig. 5) decreased antisense transcription (28). DFO also decreased antisense transcription (Fig. 5). Therefore, antisense transcripts are unlikely to play a role in the changes in N-myc expression as described. Thus, the main effect of DFO on N-myc is to block the initiation of transcription.

Inhibition of Reporter Gene Expression. To confirm that DFO inhibits N-myc promoter activity, we investigated the transient expression of an N-myc promoter (\(-1887\text{ to } +1058\) )CAT reporter construct in LA-N-1 cells. This construct was cotransfected by electroporation with PRSV.\(\beta\)-gal as a transfection efficiency control. The expression of the \(\beta\)-galactosidase gene from RSV.LTR was not affected by DFO treatment (data not shown). Immediately after transfection, cells were cultured with or without 50 \(\mu\)M DFO for 48 h, and cell lysates were analyzed for activities of CAT and \(\beta\)-galactosidase. Only 40% of the normalized CAT activity remained after DFO treatment when compared with the control (Fig. 6). Thus, DFO decreases N-myc promoter activity.

DISCUSSION

In this study, we show by TUNEL assay and DNA laddering that culturing with DFO induces apoptosis in a human neuroblastoma cell line, LA-N-1. Culturing with DFO also induces the activity of caspases, the initiator and executor enzymes of apoptosis, in LA-N-1 and additional neuroblastoma cell lines with N-myc amplification. We examined the inhibitory effect of DFO on the expression of the amplified N-myc oncogene, which is important in the biology of neuroblastomas, using Western and Northern blotting and nuclear run-on. The increase in N-myc protein by DFO is observed in LA-N-1 and five other neuroblastoma cell lines with N-myc amplification. We demonstrated that DFO also alters the transcription of some other oncogenes but not housekeeping genes. The inhibitory effect of DFO on N-myc transcription is specific inasmuch as neither actin nor GAPDH transcription is affected. Inhibition of N-myc expression appears to occur at the level of initiation rather than elongation of transcription, and no effects on N-myc protein or mRNA stability were found. As expected from the nuclear run-on data, DFO inhibits N-myc promoter activity, which was demonstrated by transient expression of an N-myc promoter-reporter plasmid.

N-myc mRNA and protein have very short half-lives (24, 25) and constitutive transcription and translation of the N-myc oncogene is required to maintain its over-expressed level in neuroblastoma cells. Inhibition of N-myc promoter activity by 60% (Fig. 6) may lead to a dramatic decrease of N-myc mRNA and protein levels over time (Figs. 2A and 4A). Our results suggest that DFO is acting by chelating iron because it results in increased cellular transferrin receptor mRNA, which is consistent with a decreased availability of cellular iron (23). Furthermore, the addition of iron, but not other metals, to the culture prevents the effects of DFO on the inhibition of N-myc expression (Fig. 2D) as well as on the inhibition of cell growth and the induction of apoptosis (Fig. 1C).

Deregulation of N-myc expression, either up- or down-regulated, has been associated with drug-induced apoptosis in neuroblastoma (29, 30), and three target genes of the closely related transcription factor c-myc (31–33) have been demonstrated to directly mediate the apoptotic effect of c-myc up-regulation. However, the similar degrees of decrease in N-myc expression by the 9-cis or all-trans isomers of retinoic acid (34) do not appear to be directly linked to their differential effects on apoptosis in neuroblastoma cells (35). Our results showed that DFO-induced apoptosis is preceded by a decrease in the endogenous expression of N-myc. However, despite this close correlation, DFO-induced apoptosis may not result directly from the decrease in N-myc expression, because similar decreased levels of endogenous N-myc expression in IMR-32 and LA-N-5 neuroblastoma cell lines (Fig. 3) do not seem to correspond with their differential degrees of DFO-induced apoptosis measured by pan-caspase activity (Fig. 1C).

We demonstrated here that DFO decreases N-myc expression specifically by inhibiting transcription initiation (Fig. 5) and by inhibiting N-myc promoter activity (Fig. 6). Although iron has been demonstrated to affect gene expression directly in several ways, none of these direct mechanisms appears to mediate the alterations in N-myc expression. First, DFO is known to affect iron dependent regulation of transferrin receptor and ferritin expression, which occur posttranscriptionally via iron regulatory elements in the untranslated regions of their mRNAs (36). Whereas the increase in transferrin receptor and the decrease in N-myc messages occurred with similar time courses (Fig. 4A) and DFO concentration (data not shown), the inhibitory effect of DFO on N-myc expression is not mediated directly by inhibiting translation initiation or by destabilizing the N-myc mRNA because DFO had no effect on the half-lives of N-myc protein or message (Table 1), and the conserved sequence of the iron regulatory elements (36) is not present in 5’ or 3’ untranslated region of the N-myc mRNA (37, 38). Second, the iron-containing heme molecule or the heme-hemoxypin complex enhances transcription of heme oxygenase-1 (reviewed in Ref. 39) or the \(MT-1\) gene (40) by binding to the promoter regions at a heme-responsive element (39) or to the heme-hemoxypin complex-related binding sites. With these mini-

Fig. 6. Inhibition by DFO of CAT reporter gene expression driven by an N-myc promoter (\(-1887\text{ to } +1058\)). Data shown were corrected by transfection efficiency in each dish and representative from one experiment with three replicates. The same experiment was repeated twice. [\(\square\)] control treated with water; [■] treated with 50 \(\mu\)M DFO; * Student’s \(t\) test, one-tailed, \(P < 0.005\).
mum elements (39) absent in the N-myc promoter or the known N-myc genomic sequence (37, 38), the inhibitory effect of DFO on N-myc transcription is unlikely to be mediated directly by the effects on iron in heme or heme-hemopexin interaction with the N-myc promoter. Instead, iron chelation may affect N-myc transcription initiation or promoter activity indirectly via the effects on iron or heme-dependent gene expression or through other effects of iron chelation.

The cellular effects of iron chelation with DFO have been attributed extensively to the inhibition of RR (8–12). However, the decrease in N-myc expression by DFO is not mediated through the inhibition of RR because hydroxyurea, a specific inhibitor of this enzyme (20), does not decrease N-myc expression in our study. Mammalian RR catalyzes the reduction of ribonucleotides to their corresponding deoxyribonucleotides. Its smaller (R2) subunit contains a binuclear ferric iron center and a tyrosyl-free radical. The latter is stabilized by the iron center and is essential for enzymatic activity (8). DFO chelates the intracellular iron pool (8), whereas hydroxurea reduces the RR iron center, inducing iron loss, and scavenges the tyrosyl radical of active R2 protein (41). As a result, both DFO and hydroxyurea prevent formation of the iron-radical center in newly synthesized apo-R2 protein, and hydroxurea removes iron from and inactivates active R2 protein (8, 41). In addition, it is not known whether the effects of DFO on the inhibition of DNA synthesis and the induction of apoptosis are mediated by the inhibition of RR. Both DFO and hydroxurea lead to the inhibition of DNA synthesis and cell growth as well as to the induction of apoptosis in mammalian cells (42, 43). They both inhibit cell growth of cultured neuroblastoma cell lines. DFO arrests cultured neuroblastoma cell lines, in a cell line-dependent fashion, predominantly in either G1 or S phase, whereas hydroxurea consistently arrests them in S phase, suggesting that DFO may have effects on cell growth in addition to its inhibition of RR. Because chelation of the intracellular iron pool by DFO may disrupt iron-dependent processes other than RR, iron chelation may lead to apoptosis by routes independent of the inhibition of RR in neuroblastoma cells.

We demonstrated that DFO treatment markedly and specifically increased the mRNA level of c-fos, but not c-jun, in LA-N-1 neuroblastoma cells (Fig. 4B). DFO treatment was also shown by others to enhance c-fos expression in another neuroblastoma cell line, IMR-32 (44). In some cell types, Fos/Jun (AP-1) transcription factor complex may function to modulate stress-induced apoptosis (45). The effects of DFO on AP-1 functional activity as well as the significance of c-fos expression on N-myc expression and apoptosis are unknown. Also, iron chelation has been reported by others to lead to an increase in p53 expression in a human leukemic cell line (46), and the p53 tumor suppressor protein may induce apoptosis via a complex network of interacting pathways. In human primary neuroblastomas, p53 is usually wild-type (47), and its signal transduction pathway is intact (48); but in some tumors it may be aberrantly translocated to the cytoplasm, thus compromising its suppressor function (49). Currently, the status of p53 in DFO-treated LA-N-1 neuroblastoma cells and its relationship to DFO-induced apoptosis is not known.

N-myc amplification results in overexpression of N-myc in human neuroblastoma. Whereas the extent of expression of the amplified N-myc genes is quite variable, DFO decreases N-myc expression in all cell lines that we examined. N-myc amplification may be responsible for treatment failure in those neuroblastoma patients receiving cisplatin or VP-16 (50). Given the clinical importance of N-myc expression, combined use of these drugs and an iron chelator to decrease N-myc expression might be clinically useful.

ACKNOWLEDGMENTS

We thank Dr. Douglas D. Ross for critical reading of this manuscript and for encouragement. We thank Dr. Paul Amstad at the Intergen Co. and Natanie M. Wehman at the Greenbaum Cancer Center for assistance with CaspTag assay activity by flow cytomtery.

REFERENCES


Inhibition of N-myc Expression and Induction of Apoptosis by Iron Chelation in Human Neuroblastoma Cells


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/3/1073

Cited articles
This article cites 47 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/3/1073.full#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/3/1073.full#related-urls

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, contact the AACR Publications Department at permissions@aacr.org.