Deferoxamine Inhibition of Human Neuroblastoma Viability and Proliferation

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ABSTRACT

Patients with widespread neuroblastoma (NB) frequently have elevated serum ferritin levels, and recently anti-NB effects of the iron chelator deferoxamine (DFO) have been reported. We have investigated the effect of DFO on human bone marrow NB cells from two untreated children with Evans Stage IV disease. DFO treatment caused dose- and time-dependent cytotoxicity of NB cells, with maximal killing at exposure to 50 μM DFO for 72 h. Cytotoxicity was prevented by cotreatment with stoichiometric amounts of iron salts and reversible by removal of DFO for 72 h. Cytotoxicity was prevented by cotreatment with iron salts. Thus, DFO has potent antitumor effects on human NB cells which appear to be related to iron deprivation. DFO should be considered for further preclinical evaluation as an anti-NB agent.

INTRODUCTION

Iron and ferritin appear to play an important role in the growth and proliferation of human NB.2 Hann and coworkers (1, 2) demonstrated that patients with advanced NB frequently have elevated serum ferritin which apparently is derived from the NB. The relationship between ferritin, NB cells, and the host is unknown, but previous studies have indicated an indirect mechanism by which ferritin inhibits host immune response, thereby enhancing tumor growth (3, 4). More recently, a direct effect on tumor growth has been supported by the demonstration that the iron chelator deferoxamine has an in vitro anti-NB effect on the human NB cell lines CHP126 and CHP100 (5). We have studied the direct effect of DFO on the viability and proliferative capacity of bone marrow NB cells obtained from two patients with widespread NB. Our results further support a role for iron in the growth and proliferation of NB, and they suggest that DFO or other iron chelators should be evaluated further for NB therapy.

MATERIALS AND METHODS

Neuroblastoma Cells. Approximately 5 ml of heparinized bone marrow were obtained from two patients, an 18-mo-old Black female (Patient A) and a 15-mo-old White male (Patient B), with Evans Stage IV NB, whose serum ferritin levels were 453 ng/ml and 592 ng/ml (normal, <140 ng/ml), respectively. Each patient had extensive bone marrow involvement. Informed consent was obtained according to institutional guidelines. Heparinized marrow was diluted in TCM (RPMI 1640; Gibco, Grand Island, NY; 10% heat-inactivated fetal calf serum, Gibco; penicillin, 50 units/ml; and streptomycin, 20 mg/ml), underlayed with Ficoll-Hypaque, and centrifuged at 1000 g for 45 min. Cells at the interface were collected, washed twice in Hanks’ balanced salt solution (Gibco), and resuspended in TCM. Cell suspensions contained >80% neuroblasts in Patient A and >90% neuroblasts in Patient B as confirmed by morphology from Wright-Giemsa-stained cytocentrifuge preparations and by neuron-specific enolase stains.

Viability of NB Cells Treated with Deferoxamine. Cells from each patient were plated in quadruplicate in 16-mm wells at 0.5 to 1.0 x 10⁵ cells/well in 1.0 ml of TCM and cultured at 37°C in 5% CO₂. Various amounts of deferoxamine mesylate (CIBA, Summit, NJ), with or without stoichiometric amounts of FeCl₃ (Sigma, St. Louis, MO), were added as indicated in “Results.” Cells were removed by scraping with a rubber policeman, and viable cells, determined by trypan blue exclusion, were counted daily for 5 days.

Clonal Growth of Neuroblastoma Cells. NB cells were cultured in 0.9% methylcellulose by a previously described method (6). Briefly, 1.0 x 10⁵ NB cells were added to 1.0 ml of 0.9% methylcellulose containing 20% α-medium (Gibco), 15% FCS, and 10% human leukocyte-conditioned medium with or without DFO and FeCl₃, as described in “Results.” Cell aggregates (>20 cells) were counted at 4, 7, and 14 days. Individual NB aggregates were removed with a micropipet and identified by Wright-Giemsa stain and by the presence of neuron-specific enolase.

RESULTS

Antineuroblastoma Effects of DFO. NB cells remained viable in suspension culture for up to 14 days, with minimal cell death over the first 5 days. Although proliferation did not occur, occasional clumps of cells would adhere to the plastic surface of the well after 2 to 3 days of culture, and thin neuritic processes would develop, often connecting with nearby clumps or single cells (Fig. 1A). Adherent cells could be disrupted easily by agitation or gentle washing. Concentrations of DFO of 5 μM or less did not affect cell viability, adherence, or morphology. At 10 μM, minimal reductions of viability were observed after 96 h (Table 1). Additionally, there was only minimal adherence to the culture surface, and the neuritic processes seen in control cultures did not occur (Fig. 1B). A dose of 20 μM led to a greater than 50% reduction in viability at 96 h, and 50 μM caused complete cell kill (Table 1). There was a slight difference in sensitivity between the patients. The effects of viability, morphology, and adherence were completely reversed by addition of stoichiometric amounts of FeCl₃.

Clonal Growth of Neuroblastoma Cells. Bone marrow NB cells grew readily in 0.9% methylcellulose supplemented with human leukocyte conditioning medium; clusters of NB cells were apparent within 72 h of plating, and aggregates (>20 clusters) developed by 14 days. In Patient A, >80% neuroblasts were observed in Patient B as confirmed by morphology from Wright-Giemsa-stained cytocentrifuge preparations and by neuron-specific enolase stains.

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Time Course of DFO Effects. Although minimal cytotoxicity occurred after 24 h at 50 μM DFO, significant anti-NB effects were observed only after 48-h exposure, with about 50% cytotoxicity at 50 μM, 30% at 20 μM, and 15% at 10 μM (Fig. 2). These values represent about one-half of eventual cell kill for each concentration. Maximal cytotoxicity occurred at 72 h, with only small increments over the next 48 h.

To evaluate the length of exposure to DFO necessary for irreversible cytotoxicity, “treatment” was stopped at 24, 48, or 72 h by washing DFO from the cultures or by supplementation with stoichiometric amounts of FeCl₃. At concentrations ≤10 μM DFO, only minimal cytotoxicity was observed even after 72-h exposure (data not shown). At higher concentrations, the cytotoxic effects of DFO were halted if washing or FeCl₃ supplementation occurred by 48 h, and no further loss of viability was observed (Table 2). Even with 50 μM DFO, cytotoxicity was reversible up to 48 h.

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: NB, neuroblastoma; DFO, deferoxamine; TCM, tissue culture medium; FCS, fetal calf serum; PHA, phytohemagglutinin; RR, ribonucleotide reductase.

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Fig. 1. Human NB bone marrow cells (1 x 10⁷/ml) were cultured in suspension as described in "Materials and Methods." After 2 to 3 days in culture, many cells became adherent and developed neuritic processes (A). Treatment with 10 µM DFO decreased adherence and prevented formation of neuritic processes (B).

**Table 1** Human bone marrow NB cells cultured at 1 x 10⁷ cells/ml at various concentrations of DFO and/or FeCl₃

Cells were counted after 96 h of continuous exposure and indicated as the percentage of viable cells compared with the total number of cells cultured. Results represent the mean of two experiments.

<table>
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<tr>
<th>Agents used</th>
<th>% of viable cells</th>
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<tbody>
<tr>
<td>DFO (µM)</td>
<td>FeCl₃ (µM)</td>
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<tr>
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<td>Patient A</td>
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<tr>
<td>0</td>
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*Mean of 2 experiments.

In some experiments, cells were exposed to various concentrations of DFO for 1 to 48 h in suspension culture, then washed twice, and plated in methylcellulose as above. There was a time- and dose-dependent effect on colony formation (Fig. 5). No reduction in aggregates was observed at doses up to 10 µM even for 48-h exposure. However, 48-h exposure to 20 or 50 µM DFO, followed by washing or FeCl₃ addition, reduced the subsequent number of aggregates at Day 14.

**DISCUSSION**

We have demonstrated that DFO has potent *in vitro* antitumor effects on human bone marrow NB cells. The effects are dose dependent, with concentrations of 10 µM being necessary for minimal killing and 50 µM for complete cell killing (>90%). At lower doses, DFO does not cause cell death but alters the morphology and decreases the ability of the NB cells to adhere to plastic surfaces. Furthermore, the effects of DFO are time dependent, with 48 to 72 h of exposure necessary to observe cytotoxicity or alterations in morphology. Removal of DFO by washing or supplementation with FeCl₃ by 48 h prevents further cytotoxicity.

Additionally, DFO inhibits the proliferation of NB cells in...
Fig. 3. Proliferation of human NB cells in methylcellulose. Bone marrow NB cells (1 x 10^6/ml) were cultured in 0.9% methylcellulose supplemented with FCS and human leukocyte-conditioned medium, as described in "Materials and Methods." Aggregates were detected by Day 5, and by Day 14 many had formed neuritic processes which often joined with other aggregates (A). Treatment with 50 μM DFO led to complete inhibition of aggregates (B).

Fig. 4. Inhibition of NB aggregates by DFO. NB cells were grown in 0.9% methylcellulose, supplemented with leukocyte-conditioned medium, as described in "Materials and Methods." DFO caused a dose-dependent reduction in the number of aggregates counted at Day 14 in each patient. Columns, mean of two experiments; bars, higher number of aggregates between the two experiments.

Fig. 5. Effect of time of exposure to DFO on proliferation. Bone marrow NB cells were exposed to various quantities of DFO for 12, 24, or 48 h, then washed 3 times, and cultured in 0.5% methylcellulose as described in "Materials and Methods." Cell aggregates were counted at Day 14 of methylcellulose culture. Columns, mean of 2 experiments; bars, higher number of aggregates between the 2 experiments.

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The effects of 50 μM DFO on proliferation were only minimally reversible after 48-h exposure to DFO followed by washing or FeCl₃ supplementation, suggesting that the viable cells remaining after a 48-h exposure to DFO may lack proliferative capacity.

Recently, Blatt and Stitely demonstrated that DFO has potent antitumor effects on two human-derived NB cell lines (5). Their results were similar to ours in that a dose of 15 μM was required for >50% cell kill, and DFO cytotoxicity was reversed by cotreatment with ferric citrate. Additionally, they demonstrated a 50% increase in the number of cells expressing the OK-T9 surface marker which is felt to be the transferrin receptor. Thus, there is evidence of iron-related anti-NB effect of DFO in both human-derived NB cell lines and in human NB cells.

The mechanism by which iron chelation inhibits NB cells is uncertain. DFO blocks the proliferation of PHA- or concanavalin A-stimulated lymphocytes and of some leukemia cell lines, apparently by preventing progression through the S phase of the cell cycle (7-11). Lederman demonstrated significant decreases in [3H]thymidine uptake in PHA-stimulated lymphocytes treated with DFO, with no significant effect on RNA or protein synthesis (7). The effect of DNA synthesis was dose dependent, was reversed when DFO was washed from culture, and was prevented by cotreatment with iron salts. Additionally, Bornford reported that DFO decreased the growth rate and inhibited DNA synthesis in the human leukemic line K562 (10).

Iron is required for the enzyme RR, important in the production of deoxyribonucleotides necessary for DNA synthesis (12). The M2 subunit of RR has a short half-life, and its regeneration requires the constant presence of iron (13-16). Inhibition of M2 regeneration by iron deprivation could decrease RR activity and thereby impede DNA synthesis. Thus, the effect of DFO on cell proliferation may be secondary to its indirect effect on RR activity. In fact, DFO potentiates inhibitors of RR (17), and effects similar to those caused by DFO have been observed.
after treatment with hydroxyurea, a known inhibitor of RR (7,9). The direct cytotoxicity of DFO on nonproliferating NB cells is not explained by this mechanism, however. The association of ferritinemia with widespread NB suggests another possible relationship between iron and NB cells which has not been fully elucidated. Further investigations of the anti-NB effects of DFO should focus on this relationship, as well as on the effects of DFO on the M2 subunit of RR in NB cells.

The demonstration of the antitumor effect of DFO on NB cell lines and NB bone marrow cells should lead to further preclinical and perhaps clinical evaluation. Previous investigators have described tumor specificity of DFO's antiproliferative activity, with no effect on the human diploid cell line WI 38 (1,18). Additionally, preliminary results in our laboratory suggest a 10-fold decrease in sensitivity to DFO in normal human bone marrow mononuclear cells compared to human NB cells (data not presented). Experience with DFO in patients requiring chronic transfusion suggests that the drug can be administered safely by various routes in relatively high doses (19). Although drug levels are not available for routine clinical use, recently Allain et al. described measuring DFO in plasma and urine by atomic absorption spectrometry (20). A single intramuscular dose of 10 mg of DFO/kg led to a peak plasma level of 15 µmol/liter in healthy, non-iron-overloaded controls, suggesting that levels in the in vitro response range (20 to 50 µM) are attainable. In fact, Estrov et al. have treated an infant with refractory leukemia with i.v. DFO (in 1-O-D-arabinofuranosylcytosine) and demonstrated in vivo and in vitro responses, without significant toxicity (21). Thus, there appears to be some potential for use of DFO as an antitumor agent.

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REFERENCES

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