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Deferoxamine Iron Chelation Increases δ -aminolevulinic Acid Induced Protoporphyrin IX in Xenograft Glioma Model

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Abstract

Exogenous administration of δ -aminolevulinic acid (δ -ALA) leads to selective accumulation of protoporphyrin IX (PpIX) in brain tumors, and has shown promising results in increasing extent of resection in fluorescence-guided resection (FGR) of brain tumors. However, this approach still suffers from heterogeneous staining and so some tumor margins may go undetected because of this variation in PpIX production. The aim of this study was to test the hypothesis that iron chelation therapy could increase the level of fluorescence in malignant glioma tumors. Mice implanted with xenograft U251-GFP glioma tumor cells were given a 200-mg/kg dose of deferoxamine (DFO), once a day for three days prior to δ -ALA administration. The PpIX fluorescence observed in the tumor regions was 1.9 times the background in animal group without DFO, and 2.9 times the background on average, in the DFO pre-treated group. A 50% increase in PpIX fluorescence contrast in the DFO group was observed relative to the control group (t-test p-value = 0.0020). These results indicate that iron chelation therapy could significantly increase δ -ALA induced PpIX fluorescence in malignant gliomas, pointing to a potential role of iron chelation therapy for more effective FGR of brain tumors.

INTRODUCTION

Malignant gliomas account for approximately 70% of primary brain tumors in the United States (1). Surgical resection plays an important role in treatment and prognosis of patients with brain tumors, with studies showing a correlation between extent of resection and patient survival (2–5). Current neurosurgical resection of malignant gliomas includes intraoperative 3-D image guidance. A limitation of current image guidance technologies is the degree of intraoperative brain shift, causing a significant registration error between patient physical space and image space (6–8). In recent years, large clinical efforts, mostly in Germany and Japan, have used fluorescence characteristics of tumors after exogenous δ -aminolevulinic acid (δ -ALA) administration to guide neurosurgical resection of brain tumors providing real-time neurosurgical guidance (9–11,3,12–15). While this technique is promising, the fluorescence signal from low-grade tumors and certain tumor types is still below detectable levels, and so methods which might increase the production of protoporphyrin IX (PpIX) generation are still needed. Two major techniques have been used to increase PpIX fluorescence: iron chelation therapy and differentiation therapy (e.g.,

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methotrexate, vitamin D) (16–22). In this paper, iron chelation to sequester away available iron in the body is tested to see if this would increase the PpIX signal in glioma tumors.

Exogenous administration of δ -ALA overloads the heme biosynthetic pathway, causing accumulation of PpIX to levels which allow visual detection using commercial surgical microscope systems. Studies have shown that PpIX accumulation is selective to high-grade brain tumor over normal tissue (23,24,12,25,26). The reasons for selective PpIX accumulation after δ -ALA administration are many and the causes likely vary with different tumor types, however in the brain the breakdown of the blood-brain barrier is a major factor in increased delivery of δ -ALA (23).

The ALA-Glioma Study group used fluorescence-guided resection (FGR) for treatment of glioblastoma multiforme, showing a highly-statistically significant difference in extent of resection between patients undergoing FGR compared to standard white-light guided resection (9,11,3). Although these studies showed an increase in extent of resection using FGR as well as a correlation between extent of resection and patient survival, studies have also shed light on a major limitation in δ -ALA-induced PpIX production for FGR: tumor margins containing diffuse tumor cells accumulate significant levels of PpIX, nonetheless these levels are not high enough to be detected with commercial surgical systems (27,10,28,14). The potential for improved detection through enhancing the signal is always present, however this will likely not be in the area of the total light intensity, but rather in the area of enhanced filtering and fluorescence detection, relative to the excitation light.

Significantly increased PpIX levels at tumor margins might lead to more effective FGR and greater extent of resection of previously undetected tumor tissue. Previous in vitro work with prostate cancer cells, brain tumor cells, adenocarcinoma cell lines (29,20), bladder and pancreatic cancer cells (30), skin cell lines (21,31,32), and in vivo work with bladder epithelium (22) and skin tumors (31) used iron chelators to increase levels of PpIX fluorescence. Iron chelation reduces the availability of free iron that would be used by the ferrochelatase enzyme for insertion into the PpIX molecule, thereby increasing the net accumulation of PpIX (33,29). In this study we used deferoxamine, a well-known iron chelator in clinical use for over 30 years (34,35). Deferoxamine is a hexadentate iron chelator originally isolated from the fungus *Streptomyces pylosus*, which forms feroxamine, a stable 1:1 chelatoriron complex, with subsequent urinary and biliary excretion. Deferoxamine is administered parenterally with reported doses from 20 mg/kg/day to 360 mg/kg/day, with a limit of 6 g/day (36,37,34,38,39). Some common adverse effects are: local skin reactions, urine discoloration, neurotoxicity, ototoxicity, and skeletal changes (36,35). In this report, the δ -ALA-PpIX fluorescence system was studied in a xenograft glioma model with ex vivo analysis of the enhancement ratio. Animals were treated with deferoxamine, to test the hypothesis that iron chelation therapy leads to significant increases in PpIX fluorescence of gliomas.

MATERIALS AND METHODS

2.1 Cell Culture

A GFP transfected U251 (U251-GFP) human glioma cell line was used in this study. Cells were cultured in Dulbecco's modified eagle's medium (Cellgro, Mediatech, Herndon, Virginia, USA) with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Georgia, USA) 1% (v/v) penicillin-streptomycin (P-S) prepared from a stock solution of 10,000 IU penicillin and 10,000 g/ml streptomycin (Mediatech). Incubation was done in a humidified environment consisting of 95% air and 5% CO₂ at 37° C.

2.2 Animal Model

This study was approved by the Dartmouth College Animal Care and Use Committee (IACUC). Intracranial implantation of U251-GFP cells was performed on 6 week-old male athymic nude mice. Mice were anesthetized using a 90:10 mg/kg ratio mixture of ketamine/xylazine and body temperature was maintained using a heating pad during anesthesia. Skull landmarks were exposed via a small scalp incision on the superior part of the cranium. A 1 mm hole in the cranium located 2 mm anterior to the bregma and 2 mm left of the midline was made with a Dremel drill. A needle was subsequently inserted 2 mm deep into the brain via stereotactic frame guidance, and 1×10^6 cells in 10 μ l of PBS were injected over a period of 5 minutes using a Hamilton syringe. The needle was retracted and skull cleaned. The drilled hole was covered with bone wax and the scalp incision closed with Vetbond tissue adhesive (J.A. Webster, Inc., Sterling, Massachusetts, USA). Mice were examined daily to ensure proper healing of the scalp.

2.3 Chelation Therapy and PpIX Fluorescence Quantification

Mice were separated into two groups: the deferoxamine (DFO) treatment group and the control group, with both receiving δ -ALA. The DFO group received 200-mg/kg of deferoxamine mesylate (Sigma, St. Louis, Missouri, USA) dissolved in 200 μ l deionized water administered i.p. at 11, 12, and 13 days after tumor implantation. An equal volume of deionized water was administered i.p. at 11, 12, and 13 days after tumor implantation to the control group. At day 14 after tumor implantation 100-mg/kg δ -ALA (Sigma) dissolved in PBS was administered to both groups i.p. Two hours after δ -ALA administration mice were sacrificed, their brains resected whole under dim lighting, and sectioned into 1 mm thick coronal slices. The sectioned faces were placed on a fluorescence plate scanner (Typhoon 9410, GE Healthcare Life Sciences) facing the scanner imaging plane. Measurement of PpIX fluorescence was done with a 633 nm excitation laser and 650 nm long pass emission filter, followed with measurements of tissue GFP fluorescence with a 488 nm laser excitation and 526 nm short pass emission filter.

2.4 Image Analysis

GFP and PpIX fluorescence contrast in brain tumors was calculated:

$$FC = \frac{SI_{\text{tumor}} - SI_{\text{bkg}}}{SI_{\text{nl}} - SI_{\text{bkg}}} \quad \text{Equation 1}$$

where FC refers to fluorescence contrast of tumor; SI_{tumor} refers to an average signal intensity of a region of known tumor tissue; SI_{bkg} refers to an average signal intensity of background; SI_{nl} refers to an average signal intensity of normal brain in the contralateral hemisphere. The coefficient of variation for PpIX fluorescence was calculated:

$$CV = \frac{SD}{SI_{\text{tumor}}} \quad \text{Equation 2}$$

where CV refers to the coefficient of variation of tumor; SD refers to the standard deviation of the signal intensity of a region of known tumor tissue. Assessment of a significant difference of the mean GFP fluorescence contrast, mean PpIX fluorescence contrast, and mean coefficient of variation in PpIX fluorescence between the control and DFO treatment groups was done using an unpaired Student's t-test. A total of ten (10) mice were studied, five (5) for each group. For each mouse, three different regions of tumor tissue were analyzed to determine the SI_{tumor} , for a total of thirty (30) different tumor tissue regions

analyzed. A p-value < 0.05 was considered statistically significant. All statistical analyses were done with Stata 10.0 (Stata Corporation, College Station, Texas, USA).

RESULTS

Following the treatment with DFO and δ -ALA, and extraction and fluorescence scanning of the samples, the regions to be quantified were identified by the GFP fluorescence, indicating positive tumor regions. The PpIX-positive fluorescent tissues co-localized to areas of GFP-positive fluorescent tissue in mice are shown for an example data set in Fig. 1. The GFP signal is only visible in areas of tumor (right slices) and the PpIX is seen in background normal brain, with highlighted areas in the tumor regions. Fluorescence intensities were quantified from the GFP positive regions and are listed in the summary data of Table 1.

The values for mean GFP fluorescence contrast, mean PpIX fluorescence contrast, and mean coefficient of variation in PpIX fluorescence contrast as well as their corresponding 95% confidence intervals for the DFO and control groups are summarized in Table 1.

The GFP levels (t-test associated p-value = 0.37) and coefficient of variation (t-test associated p-value = 0.44) were not significantly different between control and DFO treatment groups. The PpIX fluorescence values were significantly different between the groups (t-test associated p-value = 0.0020), indicating a difference exists between the control and DFO treatment groups, with a 50% increase in mean PpIX fluorescence contrast after chelation treatment (Fig. 2).

DISCUSSION

Extent of tumor resection plays an important role in prognosis and survival of patients with malignant gliomas (40,41,27,4,9,11,3) and FGR of brain tumors is a promising technology to increase the extent of surgical resection of malignant brain tumors (27,9,11,3,42,43). Nevertheless, a major limitation of the δ -ALA-PpIX system for FGR is the lack of detectable levels of PpIX fluorescence at tumor margins using commercial microscope systems (27,28,14). Therefore, methods that increase tumor PpIX contrast are critical for this procedure to gain wider utility.

Here an in vivo study was completed using deferoxamine-mediated iron chelation therapy to quantify the increase in levels of PpIX fluorescence after exogenous administration of δ -ALA in a human glioma xenograft model. Deferoxamine was used in this study because of the good safety profile and routine use in clinical practice for treatment of acute and chronic iron overload disease (44,35). No significantly adverse drug effects were noticed in the DFO group compared to the control group after deferoxamine administration over a period of three days.

Both control and DFO treatment groups demonstrated co-localization of PpIX and GFP fluorescence. A major cause of varying levels of PpIX fluorescence is the number of viable tumor cells present (e.g., tumor bulk vs. tumor margin). Although animals in this study were injected with the same batch of U251-GFP cells, differential in vivo growth of implanted cells might be a confounding factor in quantifying PpIX fluorescence. Indications were that the tumor sizes and locations were not altered by the DFO therapy. With no significant difference in GFP fluorescence contrast, viable tumor cell populations can be assumed to be approximately the same for both groups.

PpIX fluorescence contrast between control and DFO treatment groups was subsequently measured and a statistically significant difference in the mean PpIX fluorescence contrast was determined (t-test associated p-value = 0.0020). These results showed a 50% increase in

PpIX fluorescence contrast as a result of iron chelation therapy. In addition, as evidenced by the lack of a statistically significant difference in the mean coefficient of variation in PpIX fluorescence, the increase in fluorescence cannot be attributed to a more homogeneous production, but appears to be an overall increase throughout the tumor.

It was anticipated that the iron chelator, deferoxamine, would temporarily sequester available iron stores away from tumor cells, thus decreasing the ability of ferrochelatase to reduce PpIX levels (45). The exact mechanisms causing the increased PpIX fluorescence after iron chelation therapy were not investigated, but have been well studied in the past. This proof of concept study sought to establish whether a significant increase in PpIX fluorescence of a malignant glioma model occurs with iron chelation therapy, and if clinical use is warranted. An optimal deferoxamine dose to achieve maximal increase in PpIX fluorescence without significant drug side effects was not determined, but will likely be determined by human clinical factors rather than mouse tolerance levels. Relevant clinical factors affecting selection of optimal deferoxamine dose for enhancing PpIX fluorescence might include but not be limited to: adverse drug reactions, i.e., local skin reactions, organ toxicity, drug counter indications, i.e., concomitant high dose vitamin C and deferoxamine treatment, pregnancy, therapeutic benefit-to-cost ratio, and drug tolerance (35,39).

In summary, exogenous administration of δ -ALA induces selective accumulation of PpIX in brain tumor compared to normal brain tissue. Studies using the δ -ALA-PpIX system for FGR provide exciting results about increases in extent of tumor resection in malignant gliomas. Nevertheless, tumor margins are known to not display detectable levels of fluorescence using commercial surgical microscope systems. This study used iron chelation treatment in an animal model of malignant glioma, and found a 50% significant increase in PpIX fluorescence contrast. Future studies will seek to find the optimal deferoxamine dose to achieve a maximal increase in PpIX fluorescence. Further work will also test if iron chelation therapy significantly increases PpIX fluorescence in cases of gliomas without an impaired blood-brain barrier.

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Figure 1. U251-GFP coronal brain sections. (*Upper*) Representative coronal brain section of control animal with PpIX-positive fluorescent tissue co-localized to a fraction of the GFP-positive fluorescent tumor area. (*Lower*) Representative coronal brain section of DFO-treated animal with PpIX-positive fluorescent tissue co-localized with the majority of GFP-positive fluorescent tissue.

Figure 2.

Chelation treatment effects are shown with a box and whisker diagram of PpIX fluorescence contrast in tumor tissue for both control and DFO treatment groups. Unpaired Student's t-test associated p-value = 0.0020. Box and whisker diagram represents mean (✓) square; $\pm$ 1 standard deviation-top and bottom of each box, respectively; maximum and minimum-vertical bars extending from each box; n=30.

Table 1

Mean PpIX fluorescence coefficient of variation and GFP and PpIX fluorescence contrast.

	Mean	95% C.I. of the mean
<i>PpIX Coefficient of Variation</i>		
Control Group	7.4×10^{-2}	$5.1 \times 10^{-2} - 9.7 \times 10^{-2}$
DFO Group	6.3×10^{-2}	$4.1 \times 10^{-2} - 8.4 \times 10^{-2}$
<i>GFP Fluorescence Contrast</i>		
Control Group	7.6	6.1 – 9.1
DFO Group	6.9	6.0 – 7.8
<i>PpIX Fluorescence Contrast*</i>		
Control Group	1.9	1.6 – 2.3
DFO Group	2.9	2.9 – 3.4

* Statistically significant, p-value = 0.0020