Retinoblastoma Treatment: Utilization of the Glycolytic Inhibitor, 2-deoxy-2-fluoro-D-glucose (2-FG), to Target the Chemoresistant Hypoxic Regions in LHβETATAG Retinal Tumors

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PURPOSE. To analyze the effect of the glycolytic inhibitor 2-deoxy-2-fluoro-D-glucose (2-FG) on tumor burden, hypoxia, and blood vessels in LHβETATAG retinal tumors.

METHODS. Seventeen-week-old LHβETATAG retinal tumor eyes (n = 36) were treated with 2-FG and analyzed at 1 day and 1 week post a single treatment, and 1 day post a biweekly treatment for 3 weeks. Tumor sections were analyzed for hypoxia, tumor burden, and vasculature. To assess tumor burden, sections were processed for standard hematoxylin-eosin (H&E) staining. Immunofluorescent techniques were used to stain for new and mature blood vessels.

RESULTS. Hypoxia and tumor burden reduction are significantly different between the treatment schedules used (P < 0.001). Eyes treated with 2-FG for 3 weeks showed a significant decrease in hypoxia (P = 0.001) and tumor burden (P = 0.009); whereas those treated with one injection and evaluated at 1 day and 1 week postinjection did not show a decrease in either hypoxia (P = 0.373 and P = 0.782, respectively) or tumor burden (P = 0.203 and P = 0.836, respectively). When evaluating the spatial distribution of hypoxic regions in the different areas of the tumor, 2-FG showed a differential effect on hypoxia depending on the area. Hypoxia was most decreased in the base of the treated eyes with a 95% reduction (P < 0.001).

CONCLUSIONS. This is the first study to elucidate that 2-FG treatment in retinoblastoma produces an impact on hypoxia and a concomitant decrease on tumor burden. In this study, the authors validate their previous studies by revealing that glycolytic inhibitors effectively target hypoxia in retinoblastoma tumors. The future application of 2-FG as an adjuvant treatment to standard chemotherapy may enhance the treatment of retinoblastoma. (Invest Ophthalmol Vis Sci. 2012;53: 996–1002) DOI:10.1167/iosvs.11-8265

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Retinoblastoma, as the most frequent primary intraocular malignancy in children,1,2 accounts for 4% of all the pediatric malignancies in the United States.3 Survival rates now approach greater than 95%, partially due to major advances in treatment in pediatric oncology and research focus on tumor control.4 However, 80% of all the pediatric malignancies occur in developing countries, where treatment is limited and long-term survival rates are low. In addition, existing therapies for retinoblastoma (i.e., chemotherapy and focal therapies) result in unfavorable outcomes at both the systemic and local levels.4–6

The retinoblastoma tumor presents with a heterogeneous microenvironment comprised of regions with high angiogenic activity and other areas with low oxygen (O2) tension conditions, which become more pronounced during advanced disease.5 Tumor cells that survive under these hypoxic regions have been shown to be resistant to chemotherapy and radiation.6 These standard treatment modalities selectively target the rapidly dividing cell populations. Cells under hypoxia rely on anaerobic glycolysis for ATP production, a less resourceful means than oxidative phosphorylation for glucose catabolism, and their cellular metabolism slows down significantly for energy preservation. Therefore, these cells may not respond to conventional treatments such as chemotherapy, which mainly target the rapidly proliferating cells.5,7 In previous studies, we demonstrated that hypoxic cells can be killed in the LHβETATAG retinal tumor by using the glycolytic inhibitor 2-deoxy-D-glucose (2-DG). In these studies, tumor burden was significantly reduced while effectively decreasing the amount of intratumoral hypoxia.5,6,8–10 2-DG was also shown to impact angiogenesis both in vitro and in vivo.11

The glycolytic inhibitor 2-deoxy-2-fluoro-D-glucose (2-FG) has been shown to be a more potent glycolytic inhibitor than 2-DG under low O2 tension conditions in vitro.9 Glycolysis inhibition as measured by lactate levels and cytotoxicity, was found to be more pronounced using 2-FG than 2-DG in osteosarcoma (143B) growing anaerobically by treatment with the ATP synthase inhibitor oligomycin and knockout mtDNA mutant cells.9 Because standard treatment for retinoblastoma is related to a high morbidity and potential mortality, novel and adjunct therapies are needed to treat this devastating disease.12–14 We hypothesize that 2-FG effectively targets the intratumoral cells under hypoxic conditions in vivo using our LHβETATAG animal model. Therefore, the purpose of this study is to analyze the effect of the glycolytic inhibitor 2-FG on tumor burden, hypoxia, and blood vessels in LHβETATAG retinal tumors.

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**MATERIALS AND METHODS**

**LH_BETATAG Mouse Model for Retinoblastoma**

The study protocol was approved by the University of Miami Institutional Animal Care and Use Review Board Committee, and all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The LH_BETATAG transgenic mouse model used in this study has been characterized previously. This animal model develops bilateral multifocal retinal tumors that are stable and grow at a predictable rate (i.e., tumor at 4 weeks is undetectable, at 8 weeks is small, at 12 weeks is medium, and at 16 weeks is large).16

**Subconjunctival Injections of 2-FG**

Seventeen-week-old LH_BETATAG retinal tumor right eyes (n = 36) were treated and evaluated. Mice were divided into three equal groups and received periocular injections of either 2-FG (100 mg/mL; Sigma Chemical Co., St. Louis, MO) or balanced salt solution (vehicle control; BSS; Alcon Laboratories, Inc., Fort Worth, TX) for: one time and animals were euthanized 1 day posttreatment (1 d Tx); one time and animals were euthanized 1 week posttreatment (1 wk Tx); and twice a week for three weeks and animals were euthanized 1 day after the last treatment (3 wk Tx). A total volume of 20 μL was administered in each injection. To assess hypoxia, mice received 60 mg/kg of pimonidazole via intraperitoneal injection. Mice were euthanized with CO2 fumes and eyes were enucleated. Tumor sections were analyzed for hypoxia, tumor burden, and vasculature.

**Measuring Hypoxic Regions**

To assess tumor hypoxia after treatment, LH_BETATAG mice were injected intraperitoneally with a 0.16 mL suspension of pimonidazole (a drug used to detect hypoxia that penetrates all tissues, including the brain). This suspension consisted of 10 mg of pimonidazole hydrochloride (Chemicon, Temecula, CA) in 1 mL saline. Pimonidazole is known to bind to thiol-containing proteins in cells under low O2 tension. This adduct can be detected with specific antibodies and stained using immunohistochemical techniques. Animals were euthanized 2 hours after pimonidazole injection, and eyes were harvested and sectioned for histopathologic examination. Eyes were fixed with cold methanol for 10 minutes and immunostained with a directly labeled antibody recognizing pimonidazole adducts (Hyproxeprobe 1-Mab-I-FITC, clone 4.3.11.3; Chemicon) or the same concentration of a directly labeled isotype control antibody (mouse IgG1-FITC; Caltag, Burlingame, CA). Background signal intensities were minimal. All samples were normalized to intensities from isotype controls.

Both the density and the spatial distribution of hypoxic regions were analyzed independently. The density of hypoxia was measured by calculating the ratio of the amount of pixels stained with pimonidazole (i.e., marker for hypoxia) over the amount of pixels for total tumor area (100 × HPF; Adobe Photoshop CSS; Adobe, San Jose, CA). The spatial distribution of hypoxia in each tumor was performed by doing a grading analysis of different size hypoxic areas (i.e., small, medium, large, and very large) present in each intratumoral region (i.e., apex, center, lateral, and base; 100 × HPF). The different size hypoxic areas were labeled based on a cell count in the following manner: small size hypoxic areas were represented by ≤20 hypoxic cells and were assigned a value of 1; medium size hypoxic areas were represented by 21 to 40 hypoxic cells and were assigned a value of 2; large size hypoxic areas were represented by 41 to 80 hypoxic cells and were assigned a value of 4; and very large size hypoxic areas were represented by ≥81 hypoxic cells and were assigned a value of 8. A final value for each intratumoral region (e.g., apex) in all the tumors was obtained by adding all the values assigned for the different size hypoxic areas.

**Tumor Burden Measurements**

Eyes were sectioned serially and processed for standard hematoxylin-eosin (H&E) staining. Microscopic images of H&E-stained sections (8 μm sections per eye) were obtained with a digital camera at a magnification ×40. The section of the eye containing the largest cross-sectional tumor area was chosen for analysis. Tumor boundaries were traced using imaging software (Image Pro Express Software; Media Cybernetics, Silver Spring, MD). Tumor areas for all eyes were averaged, yielding an average area for each group. Tumor burden was averaged, yielding an average area for each group. Tumor burden was expressed as the tumor/globe ratio by dividing the tumor area by the area of the globe to normalize the data as previously described.

**Immunohistochemistry**

Tumor samples were frozen in OCT immediately after enucleation and serially sectioned (8 μm). Slides were fixed with methanol for 10 minutes (−20°C) and immunohistochemical analyses were performed. Mature vessels were detected with α-smooth muscle actin (α-sma) Cy3 conjugate (1:3000; Sigma Chemical Co.) which specifically binds to pericytes. Neovessels were detected with anti-endoglin (CD105 Wi, 1:500; Abcam, Cambridge, MA), which has been shown to have specificity for endothelial cells undergoing angiogenesis.20 Alexa Fluor 488 donkey anti-mouse was used as a secondary antibody for endoglin (1:500; Invitrogen, Carlsbad, CA). Omission of the primary antibody (secondary only) was used as a negative control for nonspecific binding. Cell nuclei were stained for 5 minutes with 4′,6′ diamidino-2-phenylindole (DAPI, 1:5000; Invitrogen). Blood vessel caliber analysis and grading was performed as previously described.

**Image Analysis**

Serial cross-sections of eyes containing tumors were examined for the presence of the described markers with an upright fluorescence microscope (BX51 Olympus; Olympus American Inc., Melville, NY). All images were obtained at magnification ×100 and ×200 using different filters for the DAPI, Alexa Fluor 488, and 568 signals.

**Statistical Methods**

Analysis of variance followed by two-sample t-test was used to evaluate differences between treatment groups with respect to hypoxia and tumor burden. Results are reported from untransformed data with square root P values. The differences between treatments by location in the spatial distribution of hypoxia were made using a Greenhouse-Geisser corrected repeated measures analysis of variance. Differences in the percent reduction of new vessels and mature vessels after 2-FG treatment from the vehicle control and were evaluated by one- and two-sample t-test. Values were considered significant with P ≤ 0.05.

**RESULTS**

We have previously shown that tumor growth is directly associated with advancing age in the LH_BETATAG transgenic mouse model. Additionally, we reported that large regions of hypoxia are present in advanced disease in the retinoblastoma animal model, whereas small regions of hypoxia are present in these tumors during early stages of the disease. In the present study, LH_BETATAG transgenic mice were treated with periocular injections of 2-FG following three treatment schedules: one injection and tumors were analyzed 1 d Tx; one injection and tumors were analyzed 1 wk Tx; and two injections per week for three weeks and tumors were analyzed 3 wk Tx. There was no apparent significant toxicity observed due to the drug at the dose used in the present study.

**2-FG Treatment Decreases Hypoxia in Retinoblastoma**

Hypoxia is significantly different between the treatment schedules used (P < 0.001; Figs. 1–4). Hypoxia is decreased after 2-FG treatment compared with controls (Figs. 1, 2).
Eyes treated with one injection of 2-FG and analyzed at 1 d and 1 wk did not show a statistically significant difference between the treated group and the control (\(P = 0.373\) and \(P = 0.782\), respectively). However, hypoxia is decreased substantially at 1 d post treatment after one injection.

Because we had previously observed differences in hypoxia according to the location within retinoblastoma (i.e., apex, center, lateral, base), the regional effects of 2-FG on hypoxia were evaluated. The difference among all treatment groups for hypoxia was highly significant (\(P < 0.001\); Table 2). Our results suggest that 2-FG has a differential effect on hypoxia depending on the intratumoral region analyzed. Specifically, hypoxia was decreased in the center and lateral areas of the treated eyes by 81% and 79%. Further, hypoxia was reduced in the apex and the base of the treated eyes by 88% and 95% (Fig. 5).

2-FG Causes a Concomitant Reduction in Tumor Burden in Retinoblastoma

Tumor burden is significantly different between the treatment schedules used (\(P < 0.001\); Figs. 6, 7). Eyes treated with 2-FG for 3 weeks showed a significant decrease in tumor burden (\(P = 0.009\)); whereas those treated for 1 d and 1 wk did not (\(P = 0.203\) and \(P = 0.836\), respectively; Table 1).

Impact on Vasculature after Treatment with 2-FG

The amount of blood vessel reduction after treatment with 2-FG was analyzed by doing a vessel count of small-, medium-, and large-caliber vessels. Analysis was done using mean percent differences from 100% controls. The total blood vessel percent decrease was statistically significant for both new and mature blood vessels (\(P = 0.025\) and \(P < 0.001\), respectively) associated with the use of 2-FG, with a 29% reduction of new
and 62% reduction of mature blood vessels from nontreated groups. This impact can be seen as early as 1 wk post treatment ($P < 0.001$) and continues through the entire 3 week treatment ($P < 0.001$). The total blood vessel percent reduction for all blood vessel calibers associated with 2-FG treatment was statistically significant regardless of the vessel caliber analyzed ($P = 0.05$ for small-caliber [28%], $P = 0.008$ for medium-caliber [37%], and $P = 0.05$ for large-caliber [49%]; Fig. 8).

**DISCUSSION**

During tumorigenesis, the highly metabolic tumor cells require a steady supply of O2 and nutrients to thrive and proliferate, which is usually associated with angiogenesis and regulatory growth factors. During later stages of tumor growth, a discrepancy of O2 metabolism leads to concentrated areas with lower O2 partial pressures and a disorderly microvasculature, which further produces a microenvironment depleted of O2 and blood flow. Under these harsh conditions, tumor cells experience a switch in their metabolic machinery to survive by relying on glucose consumption and anaerobic glycolysis, which is a less efficient method of ATP production than oxidative phosphorylation. To conserve energy, the hypoxic cells proliferate at a much slower rate than the typical abnormal tumor cells; therefore, becoming resistant to antiangiogenic, chemotherapeutic, and radiation therapy treatments that target the rapidly dividing cells.

Because these cells become heavily dependent on glucose consumption under anaerobic conditions, therapeutic agents that obstruct glycolysis have been explored. It has been shown that the glycolytic inhibitor 2-DG effectively targets these hypoxic cells in retinoblastoma tumors in vivo and in osteosarcoma (143B) and knockout mtDNA mutant cells in vitro. Moreover, a combination therapy of 2-DG and chemotherapy (i.e., carboplatin), significantly reduced tumor burden in LH-BETA1AG retinal tumors more efficiently than when either treatment was provided alone. Because the commercially available 2-FG was demonstrated to be a more effective glycolytic inhibitor in vitro than 2-DG in targeting hypoxic cells, in the present study we tested the efficacy of focal, periocular delivery of 2-FG in vivo in the LH-BETA1AG transgenic mouse model of retinoblastoma.

In the present study, focal delivery of 2-FG decreased hypoxia after 2-FG injections with minimal toxicities associated with the treatment. Results suggest that longer, biweekly treatments (i.e., 2-FG treatment for 3 weeks) in later stages of tumor growth produce a significant reduction on tumor hypoxia and tumor burden at the dosages used. This outcome demonstrates

**Table 1. Treatment Schedule Effect on Hypoxia and Tumor Burden**

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Group</th>
<th>Hypoxia Density (per 100×HPF) Mean (SD)</th>
<th>Hypoxia P</th>
<th>Tumor Burden (mm²) Mean (SD)</th>
<th>Tumor Burden P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 d (one inj)</td>
<td>Control</td>
<td>1.03 (1.76)</td>
<td>0.373</td>
<td>179844 (77150)</td>
<td>0.203</td>
</tr>
<tr>
<td></td>
<td>2-FG</td>
<td>0.0068 (0.0062)</td>
<td></td>
<td>106210 (43498)</td>
<td></td>
</tr>
<tr>
<td>1 wk (one inj)</td>
<td>Control</td>
<td>1.84 (0.053)</td>
<td>0.782</td>
<td>593238 (489099)</td>
<td>0.836</td>
</tr>
<tr>
<td></td>
<td>2-FG</td>
<td>2.41 (2.63)</td>
<td></td>
<td>677717 (734085)</td>
<td></td>
</tr>
<tr>
<td>3 wk (six inj)</td>
<td>Control</td>
<td>6.42 (2.77)</td>
<td>0.001</td>
<td>669250.84 (0.000022)</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>2-FG</td>
<td>1.92 (1.70)</td>
<td></td>
<td>318185.88 (0.000026)</td>
<td></td>
</tr>
</tbody>
</table>

Hypoxia and tumor burden are significantly different between the treatment schedules used ($P < 0.001$). Eyes treated with 2-FG for 3 weeks showed a significant decrease in hypoxia and tumor burden ($P = 0.001$ and $P = 0.009$, respectively).
that 2-FG is a potent glycolytic inhibitor in targeting hypoxic cells in retinoblastoma and further corroborates our previous results showing that glycolytic inhibitors can be used as adjuvant to standard treatment modalities (e.g., chemotherapy) to target the chemoresistant, hypoxic cells. Moreover, histopathological examinations of H&E staining of the LHβETATAG transgenic mouse model.30 We have previously reported that drugs implicated in glucose catabolism have an effect on angiogenesis and/or blood vessel maturation. In the present study, treatment with 2-FG effectively decreased both the percentage of new, mature, and total blood vessels in the retinal tumors of the transgenic animal model of retinoblastoma. Previous reports in

**FIGURE 5.** Spatial distribution of hypoxia after treatment with 2-FG. The spatial distribution of hypoxia in the different areas of the tumor (i.e., apex, center, lateral, base) was evaluated by counting the amount of small, medium, large, and very large areas of hypoxia in each of the intratumoral regions (i.e., apex, center, lateral, and base; 100 × HPF). Grading scale measuring the size of the hypoxic areas and the amount of these areas per intratumoral region: Small size hypoxic areas were represented by ≤20 hypoxic cells and were assigned a value of 1; medium size hypoxic areas were represented by 21 to 40 hypoxic cells and were assigned a value of 2; large size hypoxic areas were represented by 41 to 80 hypoxic cells and were assigned a value of 4; and very large size hypoxic areas were represented by ≥81 hypoxic cells and were assigned a value of 8. The difference between treated and untreated was highly significant (P < 0.001) and there is a statistically significant interaction in the differences between treatments by location (P < 0.008). 2-FG has a differential effect on hypoxia depending on the intratumoral area: apex (88%), center (81%), lateral (79%), and base (95%).

Furthermore, we analyzed the effects of 2-FG in the different areas of the tumor. As previously characterized, the basal and central regions of retinal tumors display the highest amount of hypoxia, whereas the other regions (i.e., apex, lateral) show little to no hypoxia.5 This study is the first to show that 2-FG decreases the amount of intratumoral hypoxia in vivo in all the different areas analyzed. Our results showing that the basal regions had the greatest percentage of reduced hypoxia may be explained by the findings that control eyes present with a much higher density of hypoxic cells in these areas. Further, basal regions are potentially exposed to higher drug concentrations.

A proposed mechanism of 2-FG in effectively eliminating hypoxic cells is that it interferes with glycolysis through competitively inhibiting phosphoglucone isomerase (PGI) and allosterically inhibiting hexokinase. It is hypothesized that because tumor cells have to sustain high demands of ATP necessary for their development, they express higher levels of the hexokinase but not PGI.26,27 Glycolysis inhibition seems to be a promising tool to target the hypoxic cells that are associated with chemotherapy and radiation therapy resistivity, by interfering with the glycolytic pathway through inhibition of these enzymes.5 Data indicate that the competitive inhibition of PGI occurs at a lower Km for 2-DG and perhaps 2-FG than that of hexokinase. Thus, there appears to be a hierarchy where either of these glycolytic inhibitors primarily affect PGI and only at significantly higher concentrations affect hexokinase. If indeed hexokinase is shut down then glucose-6-phosphate levels required for the functioning of the pentose phosphate shunt will be too low. If the pentose shunt is blocked effects on fatty acid synthesis and glutathione function would be expected to be detrimental to the cell.

In addition, it has also been reported that angiogenesis, the development of new blood vessels from pre-existing ones, is also affected with the glycolytic inhibitor 2-DG in the LHβETATAG transgenic mouse model.30 We have previously reported that the mammalian target of rapamycin (mTOR), which is an upstream regulator of glycolysis, targeted mature blood vessels in the same animal model.31 These facts suggest that drugs implicated in glucose catabolism have an effect on angiogenesis and/or blood vessel maturation. In the present study, treatment with 2-FG effectively decreased both the percentage of new, mature, and total blood vessels in the retinal tumors of the transgenic animal model of retinoblastoma.

**FIGURE 6.** Reduction of tumor burden after treatment with 2-FG. Tumor burden is significantly different between the treatment schedules used (P < 0.001). Eyes treated with 2-FG for 3 weeks showed a significant decrease in tumor burden (P = 0.009); whereas those treated for 1 d and 1 wk did not show a decrease in hypoxia (P = 0.203 and P = 0.836, respectively).

### Table 2. The Spatial Differential Distribution of Hypoxia through the Different Regions of the Tumor after 2-FG Treatment for 3 Weeks

<table>
<thead>
<tr>
<th></th>
<th>Apex</th>
<th>Center</th>
<th>Lateral</th>
<th>Base</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.22 (3.49)</td>
<td>8.14 (4.87)</td>
<td>2.92 (3.57)</td>
<td>9.72 (4.74)</td>
</tr>
<tr>
<td>2-FG</td>
<td>0.375 (0.744)*</td>
<td>1.56 (1.80)*</td>
<td>0.625 (0.954)*</td>
<td>0.565 (1.24)*</td>
</tr>
</tbody>
</table>

The spatial distribution of hypoxia (mean amount of hypoxia per 100 × HPF) in the different areas of the tumor (i.e., apex, center, lateral, base) was evaluated. Results suggest that 2-FG has a differential effect on hypoxia depending on the intratumoral area.

* The difference between treated and untreated was highly significant (P < 0.001) and there is a statistically significant interaction in the differences between treatments by location (P < 0.008; Greenhouse-Geisser corrected repeated measures analysis of variance).
which 2-DG showed anti-angiogenic activity in vitro in HUVEC cells was revealed to be due to 2-DG’s activity as a mannose mimetic interfering with oligosaccharide synthesis resulting in endoplasmic reticulum stress mediated apoptosis. Thus, it appeared that 2-DG’s dual activity as inhibitor of glycosylation as well as glycolysis contributed to its overall activity in reducing hypoxic tumor cells. 2-DG’s glycolytic inhibitor analog 2-FG was previously shown to be more potent in inhibiting glycolysis than 2-DG and thereby killing hypoxic cells in vitro. Because in the present study 2-FG targets both new and mature blood vessels, it is possible that 2-FG has a dual action on glycolysis and growth factors associated with blood vessel development, thus, affecting several mechanisms associated with tumor cell development under hypoxic conditions in vitro.

The present results substantiate previous in vitro results that the glycolytic inhibitor 2-FG selectively targets the chemoresistant, hypoxic cells in LH_BETA TAG retinal tumors. Because the effects of 2-FG in the present study mimic those of 2-DG, these results also validate the use of glycolytic inhibitors in the treatment of retinoblastoma. The study additionally provides the rationale for using the retinoblastoma model in the future evaluations of glycolytic inhibitors in combination with other drugs (i.e., chemotherapy and radiation therapy) in several cancers.

In conclusion, our results further demonstrate that hypoxic regions are most pronounced during later stages of the disease that develop in the LH_BETA TAG transgenic mice containing retinal tumors. This is the first study to elucidate that periocular administration of 2-FG in this animal model produces an impact on hypoxia and a concomitant influence on tumor burden control. This study correspondingly validates our previous trials revealing that glycolytic inhibitors favor the targeting of the chemoresistant, hypoxic cells in retinoblastoma tumors. The future application of periocular 2-FG as an adjuvant treatment to standard chemotherapeutic agents can be explored to potentially enhance the treatment of pediatric retinoblastoma.

References

1002 Piña et al.


