Targeting Hypoxia, a Novel Treatment for Advanced Retinoblastoma

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PURPOSE. The purpose of this study was to evaluate the presence and extent of hypoxia in murine retinoblastoma tumors and the feasibility of targeting hypoxic cells as a novel therapeutic strategy.

METHODS. Hypoxic and vascular areas in LHβETATAG mouse retinal tumors were measured using immunohistochemistry. The glycolytic inhibitor 2-deoxy-D-glucose (2-DG) was used to test the efficacy of targeting hypoxic cells in retinoblastoma. Sixteen-week-old LHβETATAG mice received injections of saline, carboplatin (31.25 μg/20 μL), 2-DG (500 mg/kg), and carboplatin (31.25 μg/20 μL) + 2-DG (500 mg/kg). Carboplatin was administered through bieweekly subcutaneous injections to right eyes only for 3 weeks. 2-DG was administered through intraocular injection three times a week for 5 weeks. Saline was administered using both methods. Eyes were enucleated at 21 weeks of age and examined for residual tumor.

RESULTS. Hypoxic regions were observed in tumors larger than 3.28 mm². When 2-DG was combined with carboplatin, a marked decrease in tumor burden was observed that was significantly more pronounced than when either agent was given alone. The hypoxic tumor cell population as measured by pimonidazole was markedly reduced by carboplatin + 2-DG (P < 0.01) and by 2-DG alone (P < 0.01), not by carboplatin alone, indicating that 2-DG effectively killed hypoxic retinoblastoma cells in vivo.

CONCLUSIONS. Treatment with glycolytic inhibitors as adjuvants to chemotherapy has the potential to increase the efficacy of chemotherapy in advanced retinoblastoma. This approach may have benefits for children with this disease and should be further investigated. (Invest Ophthalmol Vis Sci. 2008;49: 2799–2805) DOI:10.1167/iovs.08-1751

Retinoblastoma, an ocular cancer, represents one of the most common malignant tumors of childhood and affects 1 in 15,000 live births.1,2 Despite modern chemotherapy, advanced tumors prove chemoresistant, and failure rates are high. In eyes classified as group D (very large tumor burden) by the International Classification, 77% respond poorly.3 Chemotherapy failures require either radiotherapy, with an increased risk for second cancers,4,5 or permanent removal of one or both eyes.3 Even in successful cases, current chemotherapeutic regimens produce significant morbidity, including bone marrow suppression, resulting in unplanned hospitalization, transfusion, or both in up to 75% of patients.6 There is also a question of long-term secondary leukemia induction with high-dose regimens.5,7 Given the chemotherapeutic failures in advanced disease and the undesirable toxicity of current therapies, there is a critical need for less toxic adjuvant therapies for retinoblastoma.

Solid tumors often contain hypoxic regions associated with slowly proliferating cells. Given that standard chemotherapy and radiation target the rapidly dividing cells, the slower growing cells have proven difficult to kill.8 The hypoxic microenvironment renders tumor cells dependent on anaerobic glycolysis for adenosine triphosphate (ATP) production and survival, a considerably less efficient way of producing energy from glucose than oxidative phosphorylation. To meet its energy requirements, a hypoxic cell must increase the rate of glucose uptake and glycolysis. Because cells in the hypoxic portion of tumors rely on glycolysis for survival, glycolytic inhibitors such as 2-deoxy-g-glucose (2-DG) have been developed to target these cells and have shown promise as novel adjuvants to chemotherapy. 2-DG effectively reduces human lung and bone tumor burden when combined with standard chemotherapeutic drugs in mouse xenograft studies, though the effect of 2-DG on the hypoxic, slow-growing cell population of these tumors was not specifically investigated.8 However, in vitro work clearly demonstrates that 2-DG rapidly induces the death of hypoxic cells within 24 hours in a variety of tumor lines.9 It has been reported that 2-DG and other glycolytic inhibitors, such as 2-fluorodeoxy-g-glucose, block glycolysis by inhibiting hexokinase, allowing for selective killing of hypoxic tumor cells rather than normal aerobic cells based on the following two principles. First, hypoxic tumor cells take up more glucose by upregulating glucose transporters and therefore take up more 2-DG. Second, even if normal aerobic cells accumulate enough 2-DG to inhibit glycolysis, they will survive by using fat and protein oxidative phosphorylation, whereas hypoxic tumor cells are unable to use these alternative energy sources.8,9–12

Carboplatin, a cisplatin analogue, is standard therapy for retinoblastoma and is typically used in combination with other treatment modalities. Clinical studies have demonstrated that systemic carboplatin, coupled with local tumor consolidation therapy (e.g., laser therapy or cryotherapy) is an effective treatment option in children with retinoblastoma.13 However, in advanced cases of the disease, this treatment is significantly less effective.

The rationale for combining 2-DG with carboplatin in our studies was based on the hypothesis that although retinoblastoma tumors are highly vascularized, in advanced disease hy-
Hypoxic cells constitute a significant portion of the tumor, which contributes to the ineffectiveness of standard chemotherapeutic agents. In fact, Gallie et al. previously noted, while conducting studies with nude mice, the possibility of hypoxic cells in retinoblastoma tumors contributing to therapy resistance. The purpose of our study was to evaluate the presence of hypoxic cells in a transgenic model of retinoblastoma and the feasibility of targeting the hypoxic portions of the tumor with 2-DG as a therapeutic strategy.

**METHODS**

**LH<sub>BETA</sub>T<sub>AG</sub> Transgenic Mice**

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Miami. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The LH<sub>BETA</sub>T<sub>AG</sub> transgenic mouse model used in this study has been characterized previously. Small tumors by age 8 weeks, medium tumors by age 12 weeks, and large tumors that often fill the available globe space by age 16 weeks. If animals are allowed to live beyond 22 weeks of age, tumors grow outside the orbit and the eye ruptures (HB, M-EJ, TGM, unpublished observation, 2006). These transgenic mice produce heritable ocular tumors with histologic, ultrastructural, and immunohistochemical features identical to those of human retinoblastoma.

**Measuring Hypoxic Regions**

Seventy LH<sub>BETA</sub>T<sub>AG</sub> transgenic retinoblastoma mice were evaluated. To assess hypoxia during tumor development, LH<sub>BETA</sub>T<sub>AG</sub> mice and negative, litter-matched controls of 4, 8, 12, 16 and 22 weeks of age (n = 6 per group) were injected intraperitoneally with a 0.16-mL suspension of pimonidazole (a drug used to detect hypoxia that penetrates all tissues, including brain). This suspension consisted of 10 mg pimonidazole hydrochloride (Chemicon, Temecula, CA) in 1 mL saline. Pimonidazole is known to bind to thiol-containing proteins in cells under low oxygen tension. These adducts can be detected with specific antibodies and stained using immunohistochemical techniques. Animals were humanely killed 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 (hypoxyprobe 1-mAb-1-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.

Carbonic anhydrase IX was used as a secondary method of hypoxia measurement. To localize hypoxic regions within the tumor, LH<sub>BETA</sub>T<sub>AG</sub> mice of 16 and 22 weeks of age were humanely killed by cervical dislocation, and their eyes were enucleated, embedded in optimum cutting temperature (OCT) compound, frozen in liquid nitrogen and cut into 8-μm sections. Sections of the tumors were incubated over-night at 4°C with a 1:1000 dilution of anti-mouse carbonic anhydrase IX (R&D Systems, Minneapolis, MN), a monoclonal IgG antibody that recognizes mouse carbonic anhydrase IX in cells and tissues. The slides were then developed using an anti-mouse HRP-DAB cell and tissue staining kit (R&amp;D Systems). Slides were counterstained with hematoxylin (Sigma Chemical, St. Louis, MO) and examined using bright-field microscopy at low (×20) and medium (×40) power.

**Tumor Burden Measurements**

Thirty LH<sub>BETA</sub>T<sub>AG</sub> animals (60 eyes) were divided into five groups based on age. Both eyes of each mouse were enucleated and snap frozen. Eyes were sectioned serially and processed for standard hematoxylin-eosin (H&E) staining. Microscopic images of H&amp;E-stained sections (50 sections; 8-μm sections per eye) were obtained with a digital camera at a magnification of ×40. The section of the eye containing the largest cross-sectional tumor area was chosen for analysis. Tumor boundaries were traced with the use of software (Image Pro Express, Media Cybernetics, Silver Spring, MD). Tumor areas for all eyes were 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.

**Targeting Hypoxic Cells**

To assess the efficacy of targeting hypoxic cells on tumor burden, mice 16 weeks of age (n = 10 per group) were treated with saline (APP,
Schaumburg, IL), subtherapeutic carboplatin (Paraplatin; Bristol-Myers Squibb, Hillsdale, NJ), subtherapeutic carboplatin + 2-DG (Sigma-Aldrich, St. Louis, MO), or 2-DG alone. Carboplatin (31 μg, subtherapeutic dose) was administered through biweekly subconjunctival injections for 3 weeks, as previously described.22,23 2-DG (500 mg/kg) was administered through intraperitoneal injection three times a week for 5 weeks. Tumor burden was analyzed at 21 weeks of age. Carboplatin (31 μg/20 μL) solutions were freshly prepared each time. Both 2-DG and carboplatin were filtered (0.2 μm) before use. To assess hypoxia, mice received 60 mg/kg pimonidazole through intraperitoneal injection at 21 weeks of age. Eyes were enucleated 2 hours after injection, placed in OCT, snap frozen in liquid nitrogen, and sectioned. Twelve tissue sections per eye, taken from the top, middle, and bottom of the eye, were analyzed for hypoxic regions using pimonidazole immunohistochemistry. Tumor sections with the largest circumference of the tumor were analyzed for hypoxia. Twenty-four eye sections taken at regular intervals to representatively sample the whole eye were stained with H&E to evaluate tumor burden.

### Image Analysis

Serial cross-sections of eyes containing tumors were examined for the presence of the markers with a laser confocal microscope (TCP SPS; Leica Microsystems CMS GmbH, Mannheim, Germany). All images were digitally acquired and recompiled (Photoshop CS; Adobe, San Jose, CA). Sections were viewed at ×200 magnification.

### Vessel Immunofluorescence

Angiogenic and total vessel density were evaluated with immunofluorescence, as previously described.19 Briefly, total vascular endothelial cells in tumor sections were detected with the Alexa Fluor 568-conjugated lectin (Bandeiraea simplicifolia; 1:1000; Invitrogen, Carlsbad, CA). Vascular endothelial cells of newly formed angiogenic vessels were detected by immunofluorescence with a rat monoclonal anti-mouse endoglin (CD105) antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA) and a secondary Alexa Fluor 488-conjugated antibody (1:500; Invitrogen). In the same slide, immunofluorescence for pimonidazole was performed as described. The average distance between lectin- and endoglin-positive vessels to hypoxic areas was determined with image analysis software (Application Suite Advanced Fluorescence 1.5.1 Build 869; Leica), as described by Bussink et al.”24 The two most hypoxic regions within a section were identified at 200× magnification on the confocal microscope, and the images were digitally acquired. The vessel distance to the hypoxic region was measured by drawing a measuring bar from the nearest vessels to the edges of the hypoxic regions (green fluorescence) and obtaining the average of the distances from the hypoxic regions. This analysis was performed in a masked fashion and recorded for the 16- and 22-week tumors (performed on three sections for three tumors per group).

The mean density of total (lectin-positive) and new (endoglin-positive) vessels was quantified for 12-, 16-, and 22-week tumors (n = 3 per group) using a modification of standard methods.25 Briefly, digital images of a section were acquired at 200× magnification. In a masked fashion, image analysis software (Application Suite Advanced Fluorescence 1.5.1 build 869; Leica) was used to place a rectangular box with constant area (0.25-mm diameter) over the endoglin and lectin hot spot regions (two per section). The number of vessels within this region was counted, and the vessel density per square millimeter was determined. This analysis was performed on three sections of three tumors per group.

### Statistical Methods

Statistical analyses were performed by William Feuer, a biostatistician at the University of Miami Miller School of Medicine. Pimonidazole fluorescence in tumors and tumor burden analyses were investigated with two-way analysis of variance (ANOVA). Post hoc least-significant difference tests were used to evaluate differences between treatment groups. Tumor burden differences between groups were evaluated by two sample t-test.

### Results

#### Hypoxia in Advanced Retinoblastoma

Hypoxic regions were detected with pimonidazole perfusion, as described in Methods. In this transgenic model, tumor size is directly related to age.19 In preneoplastic, small, and medium-large LHβTAG retinal tumors growing in animals at 4, 8, or 12 weeks of age, few to no hypoxic regions were detected (Fig. 1A). At 12 weeks of age, these mice had tumors averaging 0.67 mm² (± 0.49 mm²) but little to no hypoxia (Fig. 1B). In contrast, in tumors from 16-week-old (Figs. 1A, 1C) and 22-week-old (Figs. 1A, 1D) mice, significant areas of hypoxia were observed (P = 0.003). The average cross-sectional area in which hypoxia was seen in these advanced tumors was 3.28 mm² (± 0.205 mm²; Figs. 2A, 2B). The intensity of the hypoxic areas was greatest in the 16-week-old animals and slightly lower in the 22-week-old mice (Figs. 1A, 1C, 1D). At 16 weeks of age, 25.7% of the tumor was hypoxic, and at 22 weeks of age 19.7% of the tumor was hypoxic (Fig. 2B). Hypoxic regions
were mainly located in central regions of the tumor (Figs. 1C, 1D). Results found for carbonic anhydrase IX staining were comparable for pimonidazole staining (Fig. 1E). Carbonic anhydrase IX has been shown to be an accurate method for detecting hypoxic regions within tissues and to be comparable to pimonidazole staining.26–28

**Relationship of Vasculature to Hypoxic Regions**

To assess the relationship between tumor vasculature and hypoxic regions, dual-labeling studies with lectin (total vasculature) and pimonidazole were performed in mice of 16 and 22 weeks of age. Lectin immunofluorescence showed that retinal tumors in L<sub>H<sub>BETA</sub>T<sub>AG</sub></sub> mice were highly vascularized around the outer edges of hypoxic regions, but few vessels were found in hypoxic regions at 16 weeks of age (Fig. 3A). The results demonstrate that hypoxic regions were not vascularized in these retinal tumors.

A possible mechanism to explain the slight decrease in hypoxic areas observed in 22- versus 16-week-old tumors is that new blood vessels are more prevalent in and around the hypoxic areas of the larger tumors. To address this possibility, dual-labeling immunofluorescence experiments were performed using pimonidazole and endoglin (CD105), a homodimer transmembrane glycoprotein, which is the most specific marker for new vessel formation.25,29–31 Using this technique, the distance between angiogenic vessels and hypoxic regions was found to be decreased in the 22- compared with the 16-week-old tumors. Sixteen-week-old tumors had angiogenic blood vessels at an average distance of 89.85 μm from the hypoxic regions compared with 49.0 μm in the 22-week-old tumors (Figs. 3B, 3C). To determine whether there was a concomitant increase in blood vessels in the 22- compared with the 16-week-old tumors, the mean density of lectin and endoglin around these hypoxic regions was quantitated. The mean total vessel density (MVD, determined by lectin staining) increased 435% in 22- compared with 16-week-old tumors (8.7 ± 0.9 vs. 2.0 ± 0.9 vessels/mm<sup>2</sup>, respectively; Table 1). Interestingly, in 12-week-old mice with no hypoxic regions, MVD and MAVD (20.0 ± 0.4 and 15.6 ± 1.4, respectively; Table 1) were higher than in the 16- and 22-week-old mice, suggesting a critical vessel density is needed to prevent hypoxia in retinoblastoma tumors.

**TABLE 1. Vessel Density**

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>Vessels (mm&lt;sup&gt;2&lt;/sup&gt; ± SD)</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>12</td>
<td>20.0 ± 0.4</td>
</tr>
<tr>
<td>16</td>
<td>5.6 ± 2.3</td>
</tr>
<tr>
<td>22</td>
<td>11.1 ± 2.3</td>
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Mean density of total (lectin-positive) and new (endoglin-positive) vessels was determined for tumors of different ages.

**2-DG Enhanced Tumor Control**

To assess the impact of targeting hypoxic cells on tumor burden, L<sub>H<sub>BETA</sub>T<sub>AG</sub></sub> mice were treated with the glycolytic inhibitor 2-DG as monotherapy and as adjuvant to carboplatin chemotherapy. Treatment with low-dose carboplatin (31 μg) monotherapy resulted in a 52% reduction (P < 0.001) in tumor size, whereas 2-DG monotherapy resulted in a 49% reduction (P < 0.001) in tumor size compared with saline-treated controls. However, when 2-DG was used as an adjuvant to low-dose carboplatin, an 86% reduction in tumor size (P = 0.007) was observed compared with controls (Fig. 4).

Treatment with intraperitoneal 2-DG was well tolerated. No significant weight loss, change in behavior, or other side effect was observed in the treated mice. Mild fibrosis was seen after subconjunctival injections of carboplatin. This fibrosis was not significant at the time of enucleation. Histologic analysis of H&E-stained sections revealed no significant necrosis within the tumor or the retina of any group, suggesting that the mechanism of tumor burden reduction was likely caused by apoptosis. No retinal toxicities of 2-DG or carboplatin were noted in any ocular tissues on histologic examination.

**2-DG Decreased Tumor Hypoxia**

To test the hypothesis that 2-DG was reducing tumor volume by killing hypoxic cells, hypoxia was measured in the treated eyes with pimonidazole. Eyes treated with 2-DG + subtherapeutic carboplatin showed a 99% decrease in hypoxic regions (P = 0.001) in comparison with the saline-treated control group. Eyes treated with 2-DG alone showed a 98% decrease in hypoxic regions (P = 0.001) in comparison with the saline-treated control group (Fig. 5). This elimination of hypoxic cells is likely a major mechanism for the reduction of tumor burden after combined 2-DG + carboplatin treatment and 2-DG monotherapy. Importantly, because approximately 26% of the tumor was hypoxic and 2-DG alone decreased tumor burden by twice this amount (50%), the mechanism of tumor burden reduction most likely included tumor growth inhibition by 2-DG in addition to its cytotoxic effect on hypoxic cells.

Interestingly, carboplatin-treated eyes also experienced reductions in hypoxia, as measured by pimonidazole staining (38%), but less than did eyes treated with 2-DG. This reduction...
in hypoxic regions was detected even though hypoxic cells have been shown to be resistant to chemotherapeutic agents. One explanation for these results is that hypoxic regions decrease concomitantly with the decrease in tumor burden. These results are also consistent with our natural history data, suggesting a minimum tumor area is necessary for hypoxia to occur in this tumor model.

**DISCUSSION**

This study is the first to show that regional hypoxia is present in advanced (16- to 22-week) tumors of a transgenic murine retinoblastoma model. In contrast, at earlier stages of the disease (12 weeks), few or no hypoxic regions were observed. Mechanistically, it is logical to speculate that cells in a hypoxic environment are heavily dependent on glycolysis and therefore undergo cell death. One mechanism by which 2-DG inhibits glycolysis is through its inhibition of hexokinase, the key enzyme catalyzing the first step of the glycolytic pathway. It is also known that hexokinase is highly expressed in malignant cells compared with normal cells, probably reflecting the necessity of high glycolytic metabolism to maintain sufficient tumor ATP supplies. As such, the use of glycolytic inhibitors, such as 2-DG, as anticancer agents might provide a preferred therapeutic selectivity. This study suggests that the inhibition of glycolysis is an effective strategy to kill cancer cells and to overcome drug resistance associated with hypoxic conditions. Phase 1 clinical trials using 2-DG in combination with docetaxel to target slow-growing hypoxic cells in various types of tumors, including lung, breast, and bone, have recently begun.

The data presented herein provide a rationale for including retinoblastoma in future evaluations of this drug.

Previous studies have shown that reduced oxygen levels upregulate HIF1α subunits, which transactivate target genes, such as vascular endothelial growth factor, that stimulate angiogenesis. Although retinoblastoma is known as a well-vascularized tumor, our studies here indicate that at a certain size or stage of tumor development, metabolic demands exceed vascular supply, resulting in hypoxic areas. Interestingly, the hypoxic regions in the 22-compared with the 16-week-old animals were found to be slightly reduced, which could be explained by increases in new blood vessel formation as measured by lectin and endoglin staining (Table 1). Thus, it is possible that the 22-week-old tumors express higher levels of HIF1α, which would account for greater new blood vessel density. Future studies are required to address this possibility.

As opposed to targeting the hypoxic areas, vessel targeting therapy has been shown to be an effective treatment for reducing tumor burden in this mouse model of retinoblastoma and is promising as future translational adjuvant therapy. It is possible, however, that vessel targeting may lead to increased selection of hypoxic cells within the tumor. Thus, the addition of glycolytic inhibitors as adjuvants to vessel targeting therapy has the potential to eliminate hypoxic regions while enhancing vascular targeting. Importantly, this strategy may be particularly advantageous in children with late-stage retinoblastoma. Adding glycolytic inhibitors along with vascular targeting agents as adjuvant therapy for retinoblastoma may benefit children with this disease, particularly those with advanced cases. Similarly, other cancers, including chemoresistant malignancies of the central nervous system, may benefit from adjuvant 2-DG glycolytic inhibitor therapy; this remains to be investigated. This is particularly true for tumors in which new, antiangiogenic, adjuvant therapies have
failed because they may have an elevated hypoxic tumor burden.

In summary, we have demonstrated that advanced transgenic retinoblastoma tumors have significant hypoxic regions that can be targeted by the glycolytic inhibitor 2-DG. Our findings indicate that when used in combination with chemotherapeutic agents such as carboplatin, 2-DG may provide a more effective treatment strategy in patients with advanced retinoblastoma.

References


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**ERRATUM**


There was an inadvertent citation of Reference 6 in the fourth sentence of the Introduction. The sentence should read, “Similarly, several risk factors (e.g., surgery at a very early age12–14 microcornea,4–7, nuclear cataract morphology,7,12, surgical technique,6 and surgery for secondary cataract6,14) have been suggested to be associated with glaucoma after cataract surgery, but with other reports not being able to find one or more of these associations.”