Increased Hypoxia following Vessel Targeting in a Murine Model of Retinoblastoma

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PURPOSE. The purpose of this study was to evaluate the effects of vessel targeting and chemotherapy agents on inducing hypoxic regions in LHBETATAG murine retinal tumors.

METHODS. Twelve- and 16-week-old LHBETATAG transgenic retinoblastoma mice were treated with peribulbar injections to the right eye only of saline (n = 42), anecortave acetate (a single injection; 500 µg/20 µL; n = 42), or carboplatin (two injections per week for 5 weeks; 62.5 µg/20 µL; n = 42). Eyes were enucleated 1 day, 1 week, and 1 month after injection. To assess hypoxia, mice received 60 mg/kg pimonidazole via intraperitoneal injection. Eyes were enucleated, and tumor sections were analyzed.

RESULTS. Levels of hypoxia significantly increase in 16-week-old animals 1 day and 1 week after treatment with anecortave acetate, a known angiostatic agent. Eyes treated with anecortave acetate showed a 28% (P < 0.001) increase in hypoxic regions in comparison with the saline-treated control group 1 day after injection and a 17% (P < 0.001) increase 1 week after injection. In early tumors of 12-week-old animals, levels of hypoxia increased by 2.0% (P = 0.011) 1 day after anecortave acetate injection compared to controls. Levels of hypoxia significantly decrease in 16-week-old animals 1 week and 1 month after treatment with carboplatin, a chemotherapeutic agent. Eyes treated with carboplatin showed a 21.7% (P = 0.017) decrease in hypoxic regions in comparison with the saline-treated control group 1 week after injection and a 4.51% (P < 0.001) decrease 1 month after injection. In early tumors of 12-week-old animals, levels of hypoxia decreased by 0.0429% (P < 0.001) 1 month after carboplatin injection compared with controls.

CONCLUSIONS. Treatment with a vessel-targeting agent results in changes in the tumor microenvironment as early as 1 day after treatment. By increasing hypoxia in tumors, vessel-targeting agents can be combined with glycolytic inhibitors which have been shown previously to target hypoxic regions in this transgenic model. This approach may have benefits for children with this disease and should be further investigated. (Invest. Ophthalmol. Vis. Sci. 2009;50:5537–5543) DOI:10.1167/iovs.09-3702

Retinoblastoma is the most common intraocular malignancy of childhood and affects 1 in 15,000 live births.1,2 In early-stage disease current treatments are effective, but they lead to a number of local and systemic complications.3–4 Advanced stages of the disease have been more difficult to treat and are associated with high failure rates. In eyes classified as Group D (very large tumor burden) by the International Classification, 77% respond poorly to current chemotherapy treatment.5 These eyes usually require further treatments, including radiotherapy, which leads to an increased risk of second cancer.6,7 or, in the most severe cases, permanent removal of one or both eyes.8 In addition, current chemotherapeutic regimens have significant morbidity, including bone marrow suppression8 and the possibility of long-term secondary leukemia induction with high-dose regimens.9–9 Given the poor response of advanced disease to current chemotherapeutic treatments and the undesirable toxicity of the current therapies, there is a critical need to develop less toxic adjuvant therapies for retinoblastoma.

Carboplatin, a DNA-damaging agent that specifically targets proliferating cells, is standard therapy for retinoblastoma. Clinical studies have demonstrated that systemic carboplatin, coupled with local tumor consolidation therapy (e.g., laser or cryotherapy) is an effective treatment option in children with retinoblastoma.10 However, in advanced cases of the disease, this treatment is significantly less effective, even when combined with vincristine and etoposide chemotherapeutic agents.

Previous studies using the LHBETATAG mouse model of retinoblastoma have shown that advanced tumors contain regions of hypoxia.11 Solid tumors often contain hypoxic regions, which are associated with slowly proliferating cells. Given that both standard chemotherapy and radiation target the rapidly dividing cells, the slower growing cells have proven difficult to kill.12 The hypoxic microenvironment renders tumor cells dependent on anaerobic glycolysis for 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-D-glucose (2-DG) have been used to target these cells and have shown promise as novel adjuvants to chemotherapy.12–16 Previous studies using the LHBETATAG mouse model of retinoblastoma have shown that the regions of hypoxia found in advanced tumors can be selectively targeted using 2-DG, a glycolytic inhibitor.11

Several treatment strategies are being investigated, including the use of vessel-targeting therapy. Vessel-targeting therapy has been shown to be an effective treatment for reducing tumor burden in the LHBETATAG mouse model and is promising as future translational therapy.17–19

Anecortave acetate is an angiostatic cortisone, a class of compounds derived from steroids but devoid of most glucocor-
ticoid activity. Experiments using the LHβTAG mouse model suggest that vascular-targeting therapy using anecortave acetate, alone or in combination with carboplatin, reduces tumor burden. Previous studies have shown that apoptosis is a mechanism for cell death in the LHβTAG mouse model of retinoblastoma undergoing focal chemotherapy or vessel-targeting therapy. Vascular targeting with antiangiogenic and angiostatic agents is emerging as a possible treatment option for retinoblastoma given the tumor's dependence on vascular supply and its potential to promote angiogenesis. Tumor burden in these mice was more markedly reduced when the vessel-targeting agent was given at 12 rather than 16 weeks of age. Results from this study suggest that vascular targeting is more effective in the treatment of small tumors harbored by younger animals and may have restricted efficacy in the treatment of large tumors, limiting the clinical efficacy of vessel-targeting therapy.

The purpose of this study was to evaluate the changes in the intensity and sizes of the hypoxic areas of the LHβTAG murine retinal tumors after treatment with either vessel-targeting or chemotherapeutic agents. Moreover, the intent of these experiments was to provide a rationale for future experiments in which either or both of these treatments would be combined with glycolytic inhibitors, which are known to target the hypoxic cell population in this transgenic model of retinoblastoma.

**METHODS**

**LHβTAG Transgenic Mice**

The study protocol was approved by the University of Miami Institutional Animal Care and Use Committee. All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The LHβTAG transgenic mouse model used in this study has been characterized previously. Briefly, a highly expressed transgene drives retinal tumor development by overexpression of the SV40 large T antigen. LHβTAG mice typically develop 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 (unpublished observation, 2002). These transgenic mice produce heritable ocular tumors with histologic, ultrastructural, and immunohistochemical features identical to those of human retinoblastoma.

**Subconjunctival Injections**

LHβTAG, transgenic retinoblastoma mice were evaluated (n = 126). The mice were divided into three groups at 12 and 16 weeks of age and received subconjunctival injections of anecortave acetate (Alcon Pharmaceuticals, Fort Worth, TX), carboplatin (Paraplatin; Bristol-Myers Squibb, Hillsdale, NJ), or saline (APP, Schaumburg, IL). Anecortave acetate (300 μg/20 μL) was administered through a single subconjunctival injection to right eyes only. Anecortave acetate does not demonstrate any significant deleterious ocular side effects that are associated with ocular glucocorticoid therapy (cataracts and elevation of intraocular pressure). Carboplatin (62.5 μg) was administered through biweekly subconjunctival injections for 3 weeks, as previously described. Saline (20 μL) was administered through subconjunctival injections. Eyes were enucleated 1 day, 1 week, and 1 month after primary injection. Carboplatin (62.5 μg/20 μL) solutions were freshly prepared each time. Carboplatin was filtered (0.2 μL) before use.

Three variables were analyzed: treatment type (anecortave acetate and carboplatin), treatment age (12 weeks and 16 weeks), and post-treatment evaluation time point (1 day, 1 week, and 1 month after treatment).

**Measuring Hypoxic Regions**

One hundred twenty-six LHβTAG transgenic retinoblastoma mice were evaluated. To assess tumor hypoxia after treatment, LHβTAG mice 12 and 16 weeks of age (n = 7 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 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 (Hypoxyprobe 1-MAB-1-FFTC, clone 4.3,11.3; Chemicon) or the same concentration of a directly labeled isotype control antibody (mouse IgG1-FFTC; Caltag, Burlingame, CA). Background signal intensities were minimal. All samples were normalized to intensities from isotype controls. The values reported indicate the percentage of pimonidazole-stained areas in the tumors.

**Tumor Burden Measurements**

Both eyes of 126 LHβTAG animals (252 eyes) were enucleated and snap frozen. Eyes were sectioned serially and processed for standard hematoxylin–cosin (H&E) staining. Microscopic images of H&E-stained sections (50 μm sections per eye) were obtained with a digital camera at a magnification of 40×. The section of the eye containingacross-sectional tumor area was chosen for analysis. Tumor boundaries were traced (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 expressed as the tumor/globe ratio by dividing the tumor area by the area of the globe to normalize the data as previously described.

**Image Analysis**

Serial cross-sections of eyes containing tumors were examined for the presence of the described 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.

**Statistical Methods**

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**

Hypoxic regions were detected with pimonidazole perfusion, as described in Methods. In this transgenic model, tumor size is directly related to age. As shown in previous studies, preneoplastic, small, and medium-large LHβTAG retinal tumors growing in animals at 4, 8, or 12 weeks of age have few to no hypoxic regions. Retinal tumors growing in animals of 16 weeks of age have significant areas of hypoxia. There were highly significant interactions between the treatment type (anecortave acetate and carboplatin), treatment age (12 weeks and 16 weeks), and posttreatment evaluation time point (1 day, 1 week, and 1 month after treatment; ANOVA). Therefore, separate comparisons were made by each treatment age and posttreatment evaluation time point for every treatment type.

Tumor burden was analyzed for all groups (Fig. 1). In the 12-week-old-treated group, there was no change in tumor area...
hypoxia was also observed at 1 day (28%; \( P < 0.001 \)) and 1 week (17%; \( P < 0.001 \)) after treatment, but no difference was seen at 1 month after treatment in the same age group (21.2%; \( P = 0.63 \); Figs. 5, 6) of the total tumor burden, an increase of 28% in comparison with the saline controls (\( P < 0.001 \)).

**DISCUSSION**

Inhibition of angiogenesis has been proposed as a therapeutic strategy for solid tumors, including retinoblastoma. Retinoblastoma tumors are highly vascularized and depend on vascular supply for viability. The capacity of these tumors to promote angiogenesis has been demonstrated. The angiogenic potential of retinoblastoma correlates with invasive growth and metastasis and is associated with poor prognosis. Although retinoblastoma is known as a well-vascularized tumor, previous studies have shown that at an advanced stage of tumor development, metabolic demands exceed vascular supply, resulting in hypoxic areas. These hypoxic re-
gions are associated with slowly proliferating cells. Given that standard chemotherapy, including carboplatin, and radiation target the rapidly dividing cells, the slower growing cells have proven difficult to kill. Knowledge of the mechanism and timing of cell death after individual treatments is essential for combined modality therapy.

In the LHETATAG model of retinoblastoma, two different treatments, a vessel-targeting agent and a chemotherapeutic agent, were effective in reducing tumor volume, both in early and advanced disease. Previous studies have shown that anecortave acetate is effective in the treatment of early-stage transgenic retinoblastoma tumors yet ineffective as a single

**Figure 3.** Tumor area in 16-week-old LHETATAG retinal tumors. The uninvolved retina appears normal, and no signs of retinal toxicity are evident. No change in tumor burden was observed at 1 day after treatment between the anecortave acetate- (D), carboplatin- (G), and saline-treated (A) groups. There was significant reduction in tumor burden at 1 week and 1 month after treatment for both anecortave acetate (E, F) and carboplatin (H, I) compared to the saline control group (B, C) ($P < 0.001$ in all four). There was a significantly higher reduction of tumor burden at 1 week and 1 month after treatment in the carboplatin-treated group (H, I) than in the anecortave acetate-treated group (E, F) ($P = 0.032$ and $P < 0.001$, respectively). Original magnifications, $\times 40$ for each group; representative H&E images.

**Figure 4.** Percentage hypoxia in anecortave acetate and carboplatin treatment groups in 12-week-old LHETATAG retinal tumors. In the AA group, a small but significant increase in hypoxia, determined by pimonidazole staining, was observed at 1 day to 2.74% of total tumor burden and a significant decrease in hypoxia at 1 month to 0.165% of total tumor burden after treatment in the 12-week-old-treated group ($P = 0.011$ and $P < 0.001$, respectively), but no difference was seen at 1 week after treatment (1.24%; $P = 0.61$) in this same age group. In the Carbo group, a significant decrease in hypoxia, determined by pimonidazole staining, was observed at 1 month after treatment in the 12-week-old-treated group to 0.0429% of total tumor burden ($P < 0.001$), and no difference was seen at 1 day (0.635%) and 1 week (0.484%) after treatment ($P = 0.85$ and $P = 0.32$, respectively).

**Figure 5.** Percentage hypoxia in anecortave acetate and carboplatin treatment groups in 16-week-old LHETATAG retinal tumors. In the AA group, a significant increase in hypoxia, determined by pimonidazole staining, was also observed at 1 day to 51.5% of total tumor burden and 1 week to 43.2% of total tumor burden after treatment in the 16-week-old-treated group ($P < 0.001$ and $P < 0.001$, respectively), but no difference was seen at 1 month after treatment (21.2%; $P = 0.63$). In the Carbo group, a significant decrease in hypoxia, determined by pimonidazole staining, was observed at 1 week to 21.7% of total tumor burden and 1 month to 4.51% of total tumor burden after treatment in the 16-week-old-treated group ($P = 0.017$ and $P < 0.001$, respectively), but no difference was seen at 1 day after treatment in the same age group (24.5%; $P = 0.73$).
**Figure 6.** Hypoxic areas in 16-week-old LHBETATAG retinal tumors. Anecortave acetate induces hypoxia in LHBETATAG tumors. Sixteen-week-old LHBETATAG mice treated with saline, anecortave acetate, or carboplatin were perfused with pimonidazole, and right eyes were sectioned and stained. There is a significant increase in the levels of hypoxia in the 1 day (D) and 1 week (E) after anecortave acetate injection groups compared with saline controls (A, B). Representative composite images of pimonidazole (green, white arrows)-stained and DAPI (blue, nuclear stain)-stained ocular sections of LHBETATAG mice treated with saline, anecortave acetate, and carboplatin at 200x magnification. Significant areas of hypoxia can be seen in the saline-treated group (A–C), with some reduction in these areas in the carboplatin monotherapy group (G–I). Significant areas of hypoxia can be seen in the anecortave acetate after 1-day (D) and 1-week (E) groups. Significant reduction in tumor burden and decrease in hypoxia can be seen in the 1 month after carboplatin (I) and 1 month after anecortave acetate (F)-treated groups.

Anecortave acetate is an antiangiogenic agent that inhibits blood vessel growth in several preclinical models of angiogenesis, including murine intraocular tumors. This antiangiogenic agent has been shown to inhibit pathologic retinal angiogenesis while not significantly affecting physiologic retinal microvasculature, and it has proven to be safe in human clinical studies. This drug may therefore hold therapeutic potential for several ocular conditions in which angiogenesis plays a critical pathophysiologic role, including intraocular tumors. However, the increased hypoxia in advanced retinoblastoma documented in previous studies may make these tumors less sensitive to vascular targeting agents. In this study, a small amount of hypoxia was seen in early transgenic retinoblastoma tumors 1 day after anecortave acetate treatment in comparison with the control, which had no significant areas of hypoxia. No hypoxia was seen 1 week and 1 month after anecortave acetate treatment in this group. However, there was a significant decrease in tumor burden after the anecortave acetate treatment in the early-stage disease. The presence of increased hypoxic areas within the tumor after anecortave acetate treatment could be attributed to the closing of angiogenic vasculature in the tumors. The increase in hypoxic areas soon after injection in both advanced tumors and small tumors without significant hypoxia is consistent with this hypothesis. We have previously shown that there is a reduction in angiogenic blood vessel staining as early as 1 day and 1 week after anecortave acetate injection. We propose that induction of hypoxia may be a mechanism of action of anecortave acetate’s reduction in transgenic retinoblastoma tumor burden. Anecortave acetate, as well as radiotherapy and chemotherapy, have been shown in previous studies to cause cell death in the transgenic mouse tumor through apoptosis.

Indeed our results demonstrate that when treated with anecortave acetate alone, hypoxic areas within the advanced retinoblastoma tumor are increased significantly 1 day and 1 week after treatment. Interestingly, hypoxia did not increase in the 16-week-old mouse 1 month after treatment with anecortave acetate, in comparison with the saline-treated control. The hypoxic regions were equivalent within these two groups. This finding could be explained by increases in new blood vessel formation over time after the anecortave acetate injection. Previous studies have shown that reduced oxygen levels up-regulate HIF1α subunits, which transactivate target genes, such as vascular endothelial growth factor, that stimulate angiogenesis. It is possible that the treated tumors express higher levels of HIF1α, leading to an increase in new blood vessels within 1 month, which would account for the lack of increase in hypoxia. It is also possible that the decrease in tumor size 1 month after treatment enables the residual (mature) vasculature to adequately supply the remaining, smaller tumor. Future studies are required to address these possibilities.
tin resulted in a significant decrease in tumor burden. Previous studies have demonstrated that the smaller the transgenic tumor, the less hypoxic its regions.\textsuperscript{11}

Vessel-targeting therapy has been shown to be an effective treatment for reducing tumor burden in the mouse model of retinoblastoma and is promising as future translational adjuvant therapy.\textsuperscript{17–19} 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, which remains to be investigated.

In conclusion, this study is the first to demonstrate the induction of hypoxia in the LH\textsubscript{BETA(TAG)} mouse model of retinoblastoma undergoing vessel-targeting therapy. Further, we have shown that there is no induction of hypoxia in tumors undergoing chemotherapy treatment. This induction of hypoxia may account for less effective tumor burden reduction in advanced retinoblastoma tumors treated with vessel-targeting therapy and may have implications in combined modality therapies using glycolytic inhibitors, such as 2-DG, for advanced retinoblastoma. Therapy using vasculature-targeting agents in combination with glycolytic inhibitors may represent a novel option in the treatment of advanced pediatric retinoblastoma. The potential of combination treatment incorporating anecortave acetate or other vessel-targeting agents may allow enhanced tumor reduction enabling a decrease in standard treatment doses for chemotherapy. We propose that treatment of advanced disease with glycolytic inhibitors as adjuvants to vessel-targeting and chemotherapeutic agents will result in enhanced tumor control because glycolytic inhibitors have been shown to specifically target hypoxic cells.\textsuperscript{11}

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References


**Increased Hypoxia in Retinoblastoma**