Focal, Periocular Delivery of 2-Deoxy-D-Glucose as Adjuvant to Chemotherapy for Treatment of Advanced Retinoblastoma

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Purpose. The aim of this study was to evaluate the changes in tumor burden and hypoxia in the $LH_{BETA}T_{AG}$ retinal tumors after treatment with a focal, single-modality, and combination therapy using periocular carboplatin and 2-deoxy-D-glucose (2-DG).

Methods. Seventeen-week-old $LH_{BETA}T_{AG}$ transgenic mice ($n = 25$) were treated with periocular injections of varying doses of 2-DG (62.5, 125, 250, 500 mg/kg) to obtain a dose response. Same-aged mice ($n = 30$) received periocular injections of saline, carboplatin, and 2-DG. Mice were divided into six groups: saline; carboplatin (31.25 μg/20 μL, subtherapeutic dose); 2-DG (250 mg/kg); 2-DG (500 mg/kg); carboplatin (31.25 μg/20 μL) and 2-DG (250 mg/kg); and carboplatin (31.25 μg/20 μL) and 2-DG (500 mg/kg). Injections were administered twice weekly for three consecutive weeks. Eyes were enucleated at 20 weeks of age, snap frozen, and analyzed for hypoxic regions and tumor volume.

Results. The difference in percentage of hypoxia after treatment with 500 mg/kg 2-DG was statistically significant from the other dose groups ($P < 0.015$). The difference in tumor burden was statistically significant from the 250 mg/kg dose ($P < 0.015$) and 500 mg/kg dose ($P < 0.001$). Highly significant differences were found between the treatment types for tumor burden, percentage of hypoxia, and pimonidazole intensity ($P < 0.001$). Tumor burden decreased significantly after all forms of treatment ($P < 0.001$); however, tumor burden became significantly more reduced after treatment with combination therapy of carboplatin and 2-DG than with either treatment alone ($P < 0.001$). The percentage of hypoxia and pimonidazole intensity decreased after treatment with 2-DG alone and in combination with carboplatin ($P < 0.001$) in all treatment groups using 2-DG regardless of the 2-DG dose used. There was no percentage reduction of hypoxia after treatment with carboplatin alone ($P = 0.25$).

Conclusions. This study demonstrates the efficacy of focal, periocular 2-DG as an adjunct to carboplatin chemotherapy to decrease both intratumoral hypoxia and tumor burden. Hypoxia is increasingly present in advanced disease of $LH_{BETA}T_{AG}$ retinal tumors. The use of glycolytic inhibitors as a therapeutic strategy has the potential to enhance current retinoblastoma treatments. (*Invest Ophthalmol Vis Sci.* 2010;51:6149–6156) DOI:10.1167/iovs.09-5033

Retinoblastoma is the most common primary intraocular malignancy in children, affecting 1 in 15,000 live births.[1,2] Significant advances in screening and treatment has prompted a shift from primary enucleation to globe preservation incorporating local tumor control. Nonetheless, enucleation is still necessary in over 20% of children with intraocular retinoblastoma associated with advanced disease.[3,4] Current treatment modalities (e.g., chemotherapy, laser, brachytherapy, and enucleation) are associated with significant morbidity and/or potential mortality.[3–8] Successful treatments with chemotherapy have been associated with problems extending from bone marrow suppression to treatment associated secondary leukemia.[9–11] Carboplatin, a *cis*-platinum analog and a standard chemotherapeutic agent in retinoblastoma treatment, targets rapidly proliferating cells and is a successful treatment for retinoblastoma when combined with local tumor consolidation therapy (e.g., laser).[12] Nevertheless, advanced stage tumors with subretinal and vitreous seeds prove to be chemoresistant.[9]

Retinoblastoma tumors contain hypoxic regions that are most prominent during advanced disease in $LH_{BETA}T_{AG}$ retinal tumors.[13] We have previously correlated the vasculature development in this disease with human retinoblastoma tumors.[14] Although hypoxia regions cannot be directly evaluated in human retinoblastoma samples by labeling thiol-containing proteins in cells under low oxygen tension,[15] ischemia can be evaluated by looking at necrosis. These hypoxic regions have been associated with slowly proliferating cells, which have been proven to be difficult to kill because chemotherapy and radiation specifically target a more rapidly dividing cell population.[16] In these hypoxic regions, tumor cells become dependent on anaerobic glycolysis for ATP production and survival, which is a significantly less efficient method than oxidative phosphorylation in generating energy from glucose. Glycolytic inhibitors such as 2-deoxy-D-glucose (2-DG) have been effectively used to target these hypoxic regions in the tumor microenvironment.[13,16–19]

Because the glycolytic inhibitor 2-DG kills the slowly proliferating cells that standard chemotherapeutic agents cannot target, a combination therapy of carboplatin and 2-DG may be expected to significantly reduce tumor burden. In fact, we have previously shown that systemic delivery of this combination therapy effectively reduces tumor burden and hypoxia in

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$LH_{BETA}T_{AG}$ retinal tumors.[13] However, these agents were delivered intraperitoneally, thus increasing the risk for nonocular complications and toxicity. It is necessary to determine the feasibility of local delivery of 2-DG, thus providing a rationale for future clinical trials involving periocular 2-DG in children with advanced retinoblastoma. The purpose of this study is to evaluate focal treatment and its impact on tumor burden and hypoxia in the $LH_{BETA}T_{AG}$ retinal tumors after treatment with a combination therapy of periocular carboplatin and 2-DG.

Materials and Methods

$LH_{BETA}T_{AG}$ Mouse Model for Retinoblastoma

The study protocol was approved by the University of Miami Institutional Animal Care and Use Review Board Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animal model used in this study, $LH_{BETA}T_{AG}$ transgenic mice, has been extensively characterized.[20] This animal model develops bilateral multifocal retinal tumors that are stable and grow at a predictable rate (i.e., tumor at 4 weeks is clinically undetectable, at 8 to 10 weeks is small, at 12 to 14 weeks is medium, and at 16 to 18 weeks is large).[21]

Dose Response of Periocular Injections of 2-DG

Seventeen-week-old $LH_{BETA}T_{AG}$ transgenic mice (n = 25) received periocular injections of saline (APP, Schaumburg, IL) and 2-DG (Sigma-Aldrich, St. Louis, MO). The mice were divided into five groups: saline, 2-DG (62.5 mg/kg), 2-DG (125 mg/kg), 2-DG (250 mg/kg), and 2-DG (500 mg/kg). A total volume of 20 μL was administered in each eye every time for all groups, and 2-DG was administered biweekly for three consecutive weeks. All solutions were filtered and freshly prepared every time. Eyes were enucleated at 20 weeks of age, snap frozen, and analyzed for hypoxic regions and tumor burden. A human clinical trial of oral delivery of 2-DG was performed, and in that trial no adverse events related to ocular function were reported specifically, including no changes in visual acuity or field.[22]

Periocular Injections of Carboplatin and 2-DG

Seventeen week-old $LH_{BETA}T_{AG}$ transgenic mice (n = 30) received periocular injections of saline, carboplatin (Paraplatin; Bristol-Myers Squibb, Hillsdale, NJ), and 2-DG. Mice were divided into six groups: saline; carboplatin (31.25 μg/20 μL, subtherapeutic dose); 2-DG (250 mg/kg); 2-DG (500 mg/kg); carboplatin (31.25 μg/20 μL) and 2-DG (250 mg/kg); and carboplatin (31.25 μg/20 μL) and 2-DG (500 mg/kg). A total volume of 20 μL was administered in each eye every time for all groups. Injections were delivered biweekly for three consecutive weeks. Eyes were enucleated at 20 weeks of age, snap frozen, and analyzed for hypoxic regions and tumor burden.

Measuring Hypoxic Regions

To assess tumor hypoxia after treatment, $LH_{BETA}T_{AG}$ mice received 60 mg/kg (0.16 mL) of pimonidazole hydrochloride suspended in saline (10 mg/mL; Chemicon, Temecula, CA) via intraperitoneal injection 2 hours before enucleation. Pimonidazole is a drug used to detect hypoxia. It ubiquitously penetrates tissues, including the eye and brain, and binds to thiol-containing proteins in cells under low oxygen tension.[15] These adducts can be detected with specific antibodies and stained using immunohistochemical techniques. After enucleation, eyes were harvested, sectioned, and fixed with cold methanol (10 minutes) for histopathologic examination. Sections containing the largest area of the tumor were 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). Cell nuclei were stained for 5 minutes with 4′, 6′ diamidino-2-phenylindole (DAPI, 1:5000; Invitrogen, Carlsbad, CA). Areas of interest within the $LH_{BETA}T_{AG}$ retinal tumors were selected blindly using DAPI staining. Only cells that had a clearly labeled nucleus with DAPI were incorporated in the analyses. The values reported indicate the percentage of pimonidazole-stained areas in the tumors.

Tumor Burden Measurements

Eyes were sectioned serially and processed for standard hematoxylin and eosin (H&E) staining. The H&E stain was performed in previously bleached tissues, and the PAS stain was modified by omitting the Harris hematoxylin or light green counterstain.[23] Microscopic images of H&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 described previously.[24]

Image Analysis

Serial cross-sections of the tumors were examined for the presence of the markers with an upright fluorescence microscope (BX51; Olympus

Table 1. 2-DG Dose Response Effects on Tumor Burden, Percentage of Hypoxia, and Pimonidazole Intensity in $LH_{BETA}T_{AG}$ Retinal Tumors

	Tumor Burden			Percentage of Hypoxia			Pimonidazole Intensity	
Treatment	**Mean (SD)**	***P***	**% Reduction**	**Mean (SD)**	***P***	**% Reduction**	**Mean (SD)**	***P***
Saline	5.03 (0.12)			20.1 (1.21)			59.4 (2.90)	
Saline vs. 2-DG 62.5	4.94 (0.06)	>0.2	1.83	2.46 (0.70)	**<0.001**	87.7	2.06 (0.52)	**<0.001**
Saline vs. 2-DG 125	4.87 (0.16)	>0.2	3.13	2.02 (0.26)	**<0.001**	89.9	1.92 (0.27)	**<0.001**
Saline vs. 2-DG 250	4.54 (0.29)	**<0.015**	9.74	2.04 (0.17)	**<0.001**	89.8	1.69 (0.74)	**<0.001**
Saline vs. 2-DG 500	3.87 (0.10)	**<0.001**	23.1	1.38 (0.66)	**<0.001**	93.1	1.95 (0.56)	**<0.001**
Treatment	**Mean Difference**	***P***	**% Reduction**	**Mean Difference**	***P***	**% Reduction**	**Mean Difference**	***P***
2-DG 62.5 vs. 125	−0.065	>0.05	1.3	−0.43	>0.15	17.5	−0.14	>0.3
2-DG 62.5 vs. 250	−0.397	**<0.015**	8.0	−0.42	>0.15	17.1	−0.37	>0.3
2-DG 62.5 vs. 500	−1.07	**<0.001**	21.7	−1.07	**<0.015**	43.5	−0.11	>0.3
2-DG 125 vs. 250	−0.332	**<0.015**	6.8	0.017	>0.15	−0.8	−0.23	>0.3
2-DG 125 vs. 500	−1.00	**<0.001**	20.5	−0.64	**<0.015**	31.5	0.03	>0.3
2-DG 250 vs. 500	−0.67	**<0.001**	14.8	−0.66	**<0.015**	32.3	0.26	>0.3

One-way ANOVA and least significant difference tests were performed for the multiple comparisons. In bold, $P < 0.05$ statistically significant values.

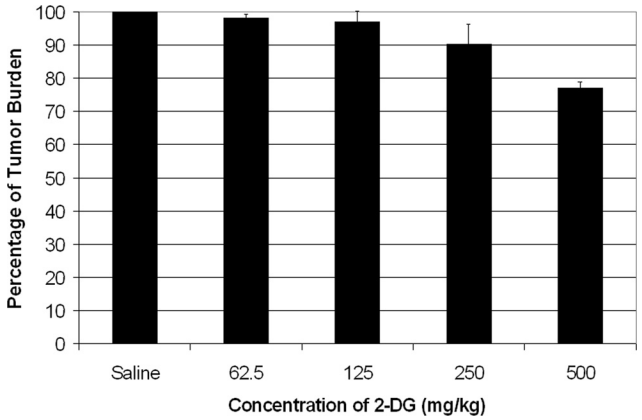

FIGURE 1. Effect of 2-DG on tumor burden. Various doses of 2-DG plotted versus percentage of tumor burden compared to saline controls. Only the higher doses of 2-DG have a significant effect on tumor control, with the highest doses (250 and 500 mg/kg) corresponding to the greatest reduction in tumor burden (9.7% and 23%, $P < 0.015$, $P < 0.001$, respectively).

American, Melville, NY). All images were obtained at 200× magnification using different filters for the DAPI, Alexa Fluor 488, and 568 signals. Measured parameters (e.g., number of hypoxic cells, pimonidazole intensities) were evaluated as the average from at least five different adjacent sections per tumor per eye. The results from all sections were averaged.

Statistical Methods

Pimonidazole fluorescence in tumors and tumor burden analyses were investigated with one-way ANOVA for 2-DG dose–response groups and two-way ANOVA for carboplatin and 2-DG treatment groups. Post hoc least-significant difference tests were used to evaluate differences between 2-DG dose–response groups and carboplatin and 2-DG treatment groups. Tumor burden differences between groups were evaluated by a two-sample *t*-test. Results were considered significant if $P \leq 0.05$. Confidence intervals (CIs) were included for every *P* value obtained.

RESULTS

Tumor growth is directly associated with advancing age in the $LH_{BETA}T_{AG}$ transgenic mouse model.[25] We have previously shown that hypoxia is significantly detected in large-size $LH_{BETA}T_{AG}$ retinal tumors, and minimal hypoxia is observed in small $LH_{BETA}T_{AG}$ retinal tumors.[13]

Dose Response of Periocular Injections of 2-DG

To assess the impact of periocular administration of 2-DG on tumor burden and hypoxia, $LH_{BETA}T_{AG}$ mice were treated with varying dosages of the glycolytic inhibitor. Table 1 presents the effects of the dose response of 2-DG on three measured variables in $LH_{BETA}T_{AG}$ retinal tumors: tumor burden, percentage of hypoxia, and pimonidazole intensity. There were highly significant interactions between the treatment type for tumor burden, percentage of hypoxia, and pimonidazole intensity ($P < 0.001$; ANOVA).

2-DG Dose Response on Tumor Burden. Tumor control was not different ($P > 0.2$) between the controls and the lowest two doses (62.5 and 125 mg/kg), with tumor burden values of 4.94 and 4.87 mm^2 (1.3% and 3.1% reduction). However, the difference in tumor burden was statistically significant from 250 mg/kg dose ($P < 0.015$) and 500 mg/kg dose ($P < 0.001$; Figs. 1 and 2), with tumor burdens of 4.5 and 3.9 mm^2, for a 9.7 and 23% decrease, respectively. Additionally, the difference in tumor control was significant between 250 and 500 mg/kg ($P < 0.001$).

2-DG Dose Response on Hypoxia. At all doses of periocular 2-DG (62.5, 125, 250, 500 mg/kg), both the percent hypoxia and pimonidazole intensity after drug treatment were significantly lower relative to saline controls ($P < 0.001$; Figs. 3 and 4). Saline-injected eyes demonstrated tumors with 21% hypoxia, which is consistent with our previous reports of hypoxia varying between 20% and 26%.[13] The lowest dose of 2-DG (62.5 mg/kg) significantly decreased hypoxia to 2.4%, compared with 21% in controls. The highest dose of 2-DG (500 mg/kg) significantly decreased hypoxia to 1.4%, compared with 21% in controls. When hypoxia after drug treatment was analyzed between each drug treatment group, the 62.5, 125, and 250 mg/kg doses did not differ from each other ($P > 0.15$). Additionally, the difference in pimonidazole intensity after each drug dosing group was not statistically significant ($P > 0.3$). However, the difference in percentage of hypoxia after treatment with 500 mg/kg was statistically significant from the other drug treatment groups ($P < 0.015$). Figure 5 shows a dose-dependent response of 2-DG on tumor hypoxia, with the highest dose (500 mg/kg) significantly reducing hypoxia by 93% compared with the lowest dose (62.5 mg/kg) of 87% ($P < 0.015$).

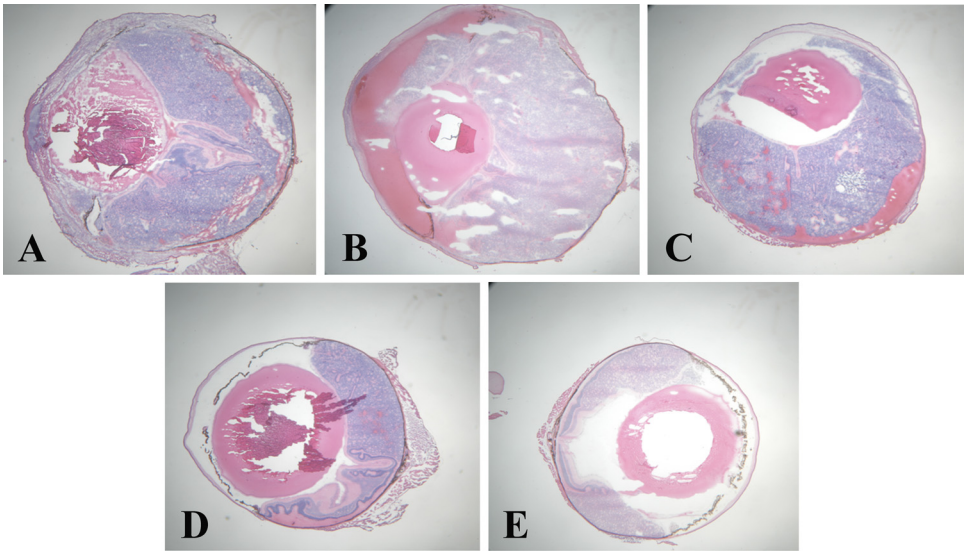

FIGURE 2. Histopathology of $LH_{BETA}T_{AG}$ retinal tumors treated with varying doses of 2-DG. 2-DG enhances tumor control at all periocular doses, with the highest doses (250 and 500 mg/kg) producing a significant decrease in tumor burden compared to control (9.7% and 23%, respectively). (**A**) Saline control, (**B**) 2-DG at 62.5 mg/kg, (**C**) 2-DG at 125 mg/kg, (**D**) 2-DG at 250 mg/kg, and (**E**) 2-DG at 500 mg/kg. Representative H&E images of each group at 40× magnification.

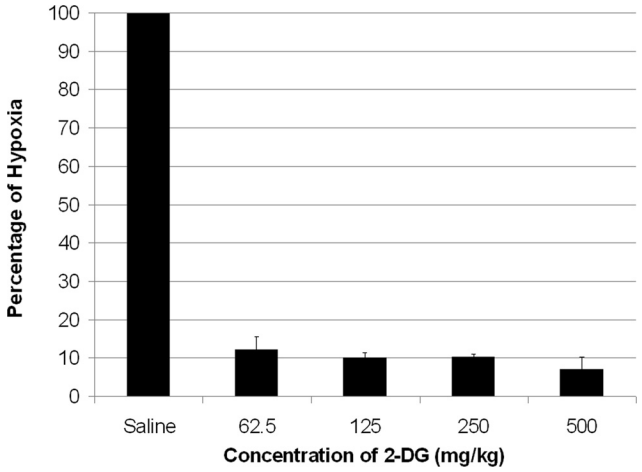

FIGURE 3. Effect of 2-DG on tumor hypoxia: varying doses of 2-DG plotted versus percentage of total tumor hypoxia. All doses of 2-DG have a significant effect on tumor hypoxia compared to saline controls ($P < 0.001$).

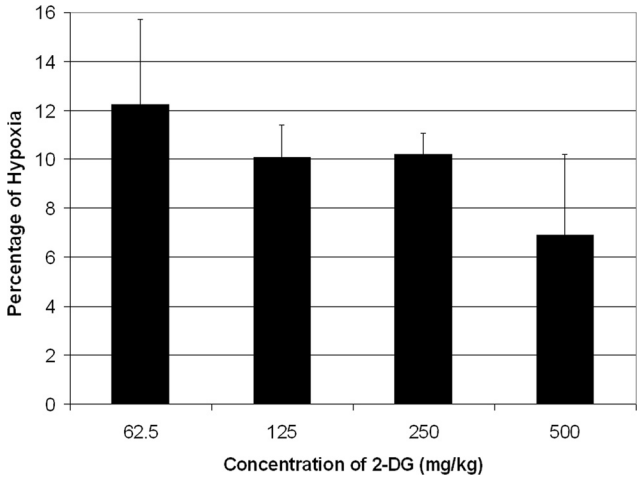

FIGURE 5. Effect of 2-DG on tumor hypoxia: varying doses of 2-DG plotted versus percentage of hypoxia remaining compared to saline control. 2-DG has a dose-dependent effect on decreasing tumor hypoxia, with higher doses (500 mg/kg) exerting a significantly greater effect (93.1% reduction versus 87%) compared with lower doses (62.5 mg/kg; $P < 0.015$).

Periocular Injections of Carboplatin and 2-DG

Table 2 presents the effects of the treatment types delivered (i.e., carboplatin and 2-DG) on three measured variables in $LH_{BETA}T_{AG}$ retinal tumors: tumor burden, percentage of hypoxia, and pimonidazole intensity. There were highly significant interactions between the treatment type for tumor burden, percentage of hypoxia, and pimonidazole intensity ($P < 0.001$; ANOVA).

Tumor Burden Reduction after Treatment with 2-DG and Carboplatin. Tumor burden decreased significantly after treatment with periocular carboplatin alone ($P < 0.001$), periocular 2-DG alone (250 mg/kg; $P = 0.04$), 2-DG (500 mg/kg; $P < 0.001$), and periocular carboplatin combined with both doses of 2-DG ($P < 0.001$; Figs. 6 and 7). Greatest tumor burden reduction was noted after treatment with combination therapy of carboplatin and 2-DG than with either treatment alone (i.e., carboplatin or 2-DG; $P < 0.001$).

Hypoxia Changes after Treatment with Carboplatin and 2-DG. The percentage of hypoxia decreased significantly after treatment with 2-DG alone and in combination with carboplatin ($P < 0.001$) in all treatment groups using 2-DG regardless of the 2-DG dose used (Figs. 8 and 9). There was no hypoxia percentage reduction after treatment with carboplatin alone ($P = 0.25$). Similarly, the pimonidazole intensity decreased significantly after treatment with 2-DG alone and in combination with carboplatin ($P < 0.001$) in all treatment groups using 2-DG regardless of the 2-DG dose used.

Discussion

Developing tumors rely on highly proliferating cells and an evolving tumor microenvironment including ongoing angiogenesis to grow and survive.[26] Many of the regulatory factors expressed during tumor growth have been explored to therapeutically target solid tumors, including retinoblastoma.[27–34] Carboplatin, a chemotherapeutic agent that targets the rapidly dividing cells, has been successful in the treatment of retinoblastoma.[12] Nevertheless, advanced stage tumors prove to be

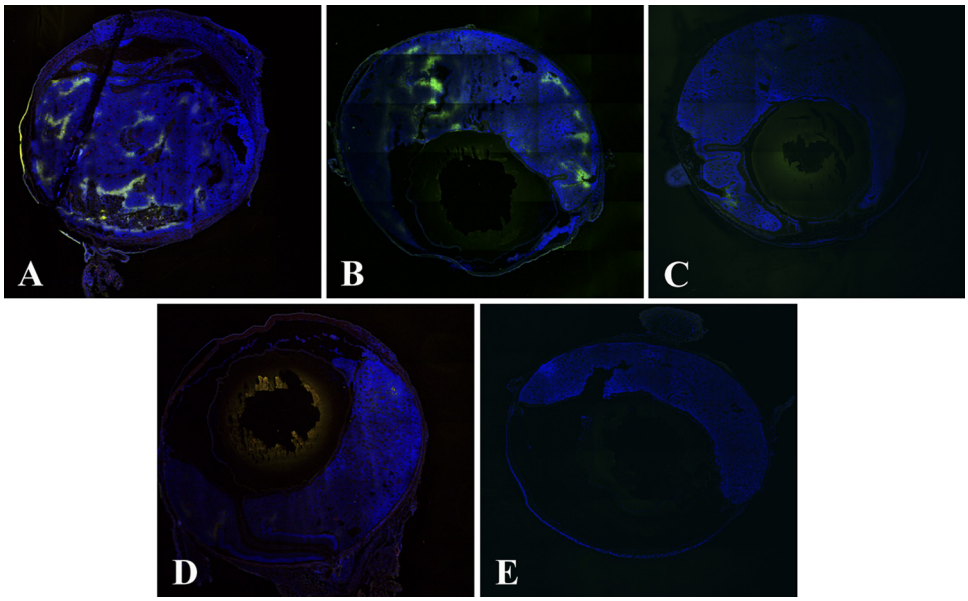

FIGURE 4. Pimonidazole intensity in tumors treated with varying doses of 2-DG. 2-DG significantly decreased pimonidazole intensity, correlating with a decrease in hypoxia. (**A**) Saline control, (**B**) 2-DG at 62.5 mg/kg, (**C**) 2-DG at 125 mg/kg, (**D**) 2-DG at 250 mg/kg, (**E**) 2-DG at 500 mg/kg. Representative composite images of pimonidazole (*green*) and DAPI (*blue*)-stained ocular sections of $LH_{BETA}T_{AG}$ mice.

TABLE 2. Treatment Type Effects on Tumor Burden, Percentage of Hypoxia, and Pimonidazole Intensity in $LH_{BETA}T_{AG}$ Retinal Tumors

Treatment	Tumor Burden			Percentage of Hypoxia			Pimonidazole Intensity		
	Mean (SD)	*P*	CI	Mean (SD)	*P*	CI	Mean (SD)	*P*	CI
Saline vs. Carbo	1.69 (0.11)	**<0.001**	1.41 to 1.97	1.35 (1.05)	0.25	−1.21 to 3.91	16.41 (2.37)	**<0.001**	10.61 to 22.20
Saline vs. 2-DG 250	0.49 (0.16)	**0.04**	0.05 to 0.93	18.05 (0.61)	**<0.001**	16.15 to 19.96	57.71 (1.5)	**<0.001**	54.05 to 61.38
Saline vs. 2-DG 500	1.16 (0.07)	**<0.001**	0.99 to 1.33	18.71 (0.63)	**<0.001**	17.23 to 20.20	57.45 (1.31)	**<0.001**	54.37 to 60.54
Saline vs. Carbo+2-DG 250	2.2 (0.12)	**<0.001**	1.90 to 2.50	18.82 (0.77)	**<0.001**	16.83 to 20.81	57.58 (1.76)	**<0.001**	53.05 to 62.11
Saline vs. Carbo+2-DG 500	3.31 (0.22)	**<0.001**	2.79 to 3.82	19.69 (0.61)	**<0.001**	17.77 to 21.61	57.88 (1.29)	**<0.001**	54.82 to 60.94
Carbo vs. 2-DG 250	1.2 (0.18)	**<0.001**	0.77 to 1.63	−16.71 (0.86)	**<0.001**	−19.41 to −14.00	−41.31 (1.91)	**<0.001**	−45.97 to −36.64
Carbo vs. 2-DG 500	0.53 (0.1)	**0.001**	0.29 to 0.76	−17.36 (0.82)	**<0.001**	−19.31 to −15.42	−41.05 (1.67)	**<0.001**	−44.99 to −37.11
Carbo vs. Carbo+2-DG 250	0.51 (0.15)	**0.02**	0.13 to 0.89	17.47 (1.05)	**<0.001**	14.76 to 20.18	41.18 (2.25)	**<0.001**	35.40 to 46.95
Carbo vs. Carbo+2-DG 500	1.62 (0.23)	**<0.001**	1.08 to 2.15	18.34 (0.85)	**<0.001**	15.63 to 21.06	41.48 (1.66)	**<0.001**	37.56 to 45.40
2-DG 250 vs. Carbo+2-DG 250	1.71 (0.2)	**<0.001**	1.20 to 2.22	0.76 (0.31)	0.06	−0.04 to 1.57	−0.13 (0.59)	0.83	−1.64 to 1.38
2-DG 500 vs. Carbo+2-DG 500	2.15 (0.19)	**<0.001**	1.71 to 2.59	0.98 (0.3)	**0.03**	0.15 to 1.80	0.43 (0.32)	0.22	−0.31 to 1.17

One-way ANOVA and least significant difference tests were performed for the multiple comparisons. In bold, $P < 0.05$ statistically significant values.

FIGURE 6. Tumor burden reduction after treatment with carboplatin and 2-DG. Tumor burden decreased significantly after treatment with carboplatin alone ($P < 0.001$), 2-DG alone (250 mg/kg; $P = 0.04$), 2-DG (500 mg/kg; $P < 0.001$), and carboplatin combined with both doses of 2-DG ($P < 0.001$). Comparison between treatment modalities yielded that tumor burden statistically decreased more after treatment with carboplatin than with either dose of 2-DG ($P < 0.001$). However, combination therapy caused a more statistical significant reduction of tumor burden than either treatment alone (i.e., carboplatin or 2-DG; $P < 0.001$).

chemoresistant.[9] Regulatory factors that take over during later stages of the disease coincide with the metabolic demands of blood flow and oxygen supply in most solid tumors,[35,36] resulting in the development of hypoxic regions in the tumor.[13] These hypoxic regions are associated with slowly proliferating cells, which are resistant to anti-angiogenic, chemotherapy, and radiation therapy. To adapt to reduced oxygen tensions, slow growing tumor cells become heavily dependent on anaerobic glycolysis and, thus, glucose consumption. The glycolytic inhibitor 2-DG has been shown to effectively target these hypoxic cells.[13,19]

We have previously shown that a combination therapy of systemic 2-DG and the chemotherapeutic agent carboplatin significantly reduced tumor burden in $LH_{BETA}T_{AG}$ retinal tumors more effectively than when either treatment was provided alone.[13] However, these treatments were delivered via intraperitoneal injections increasing the risk for systemic complications. Focal therapies have not been associated with any systemic toxicities including myelo suppression, fever of unknown origin, or failure to thrive. In the present study, we tested the efficacy of the carboplatin and 2-DG combination therapy provided periocularly to enhance ocular delivery and to avoid the morbidity potentially associated with systemic delivery.

We primarily provided varying doses of periocular 2-DG to the $LH_{BETA}T_{AG}$ transgenic mice to obtain the dose effect with the maximum reduction on tumor burden and hypoxia. The present study shows that 2-DG can be effectively administered locally with periocular injection. Histopathologic analysis did not reveal any local toxicities or abnormalities of the treated eyes. Results indicate that biweekly, periocular injections of 2-DG for 3 weeks in advanced retinoblastoma tumors have a dose–response effect on tumor hypoxia and tumor burden, with greater doses (250 and 500 mg/kg) showing a greater effect. All doses had a significant effect on tumor hypoxia, with the highest dose decreasing hypoxia to 1.4% of tumor area (93% reduction significantly different from lower doses) compared with 21% in controls. Although higher doses (250 and 500 mg/kg) significantly reduced tumor burden by 9.7 and 23%, the lower doses of 2-DG (62.5 and 125 mg/kg) had a

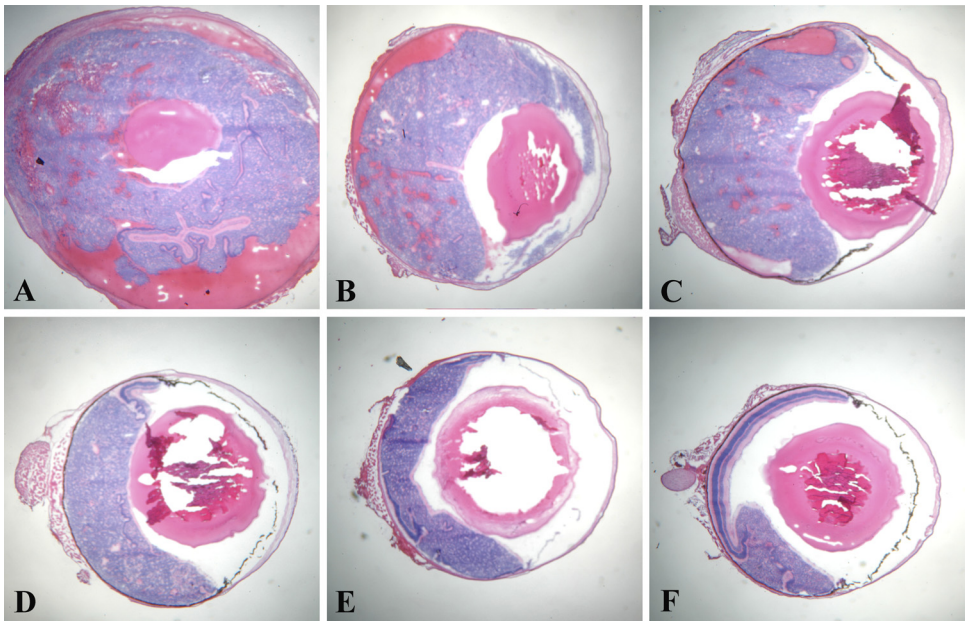

FIGURE 7. H&E of tumor burden reduction after treatment with carboplatin and 2-DG. (**A**) Saline-treated control group. (**B**) 250 mg/kg dose of 2-DG–treated eyes. (**C**) 500 mg/kg dose of 2-DG–treated eyes. (**D**) Carboplatin-treated eyes (31.25 μg/20 μL). (**E**) Carboplatin and 250 mg/kg dose of 2-DG–treated eyes. (**F**) Carboplatin and 500 mg/kg dose of 2-DG–treated eyes.

minimal effect on tumor burden, with a decrease of only 2% and 3%, respectively.

Sequentially, we tested the effects of the combination therapy of periocular carboplatin and 2-DG in $LH_{BETA}T_{AG}$ retinal tumors, using the higher doses (250 and 500 mg/kg) of 2-DG, which significantly reduced tumor burden. As expected, tumor burden decreased after periocular delivery of either carboplatin or 2-DG alone (Table 2; Figs. 6 and 7). Furthermore, we found that tumor burden reduction was significantly enhanced when both drugs were provided as a combination therapy in vivo. This result further supports and demonstrates that glycolytic inhibitors can be used as adjuvant to chemotherapy to target the chemoresistant, hypoxic cells. In addition, histopathological examinations of H&E staining of the $LH_{BETA}T_{AG}$ retinal tumor sections did not reveal any local toxicity or abnormality in the eye, further demonstrating that periocular delivery of these drugs is a feasible and effective treatment modality (Fig. 7).

As previously described, hypoxic regions are largely present in advanced disease of $LH_{BETA}T_{AG}$ retinal tumors.[13] These hypoxic regions have been formerly associated with slowly proliferating cells that have been proven to be chemoresistant in later stages of tumor development. In the present study, periocular carboplatin provided alone did not have any effect in the percentage of hypoxia present in $LH_{BETA}T_{AG}$ retinal tumors, whereas 2-DG caused a significant reduction of hypoxia. This study is part of the first series of experiments[13,19] that uses 2-DG to effectively eradicate the hypoxic cells in the tumor in vivo that appear to be resistant to chemotherapy. Our results further demonstrate that a glycolytic inhibitor is a necessary component of therapeutic agents to target tumor cells and eradicate tumor burden completely. Optimal scheduling and dosing of carboplatin and 2-DG will be analyzed in the future to totally diminish tumor burden.

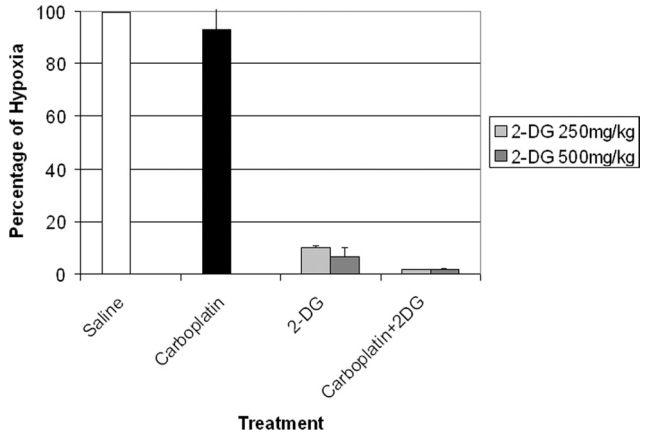

FIGURE 8. Percentage of hypoxia reduction after treatment with carboplatin and 2-DG. The percentage of hypoxia decreased significantly after treatment with 2-DG alone and in combination with carboplatin ($P < 0.001$) in all treatment groups using 2-DG regardless of the 2-DG dose used. There was no hypoxia percentage reduction after treatment with carboplatin alone ($P = 0.25$).

In the present study, approximately 20% of the tumor was composed of hypoxic cells and 2-DG alone effectively reduced tumor size by 23.1% by killing these cells and hindering tumor cell growth. Similar to our previous finding from the treatment with intraperitoneal 2-DG,[13] in the present study, periocular delivery of 2-DG alone killed hypoxic cells by 15-fold (i.e., 1.38% of hypoxia after treatment with 2-DG), eradicating almost all hypoxia present in the tumor. This result supports our initial proposal that 2-DG targets hypoxic cells and successfully decreases tumor size by killing these hypoxic cells and hindering tumor cell growth.[13,19]

Moreover, periocular delivery of carboplatin alone reduced tumor size by 33.6%, whereas 2-DG adjuvant to carboplatin reduced tumor size by 65.8%. Because the combination therapy of carboplatin and 2-DG reduced tumor burden by twice the amount of either treatment alone (i.e., carboplatin or 2-DG) and killed hypoxic cells as much as with 2-DG treatment alone, the means by which tumor burden diminishes incorporated the elimination of hypoxic cells and the inhibition of tumor growth by 2-DG. Earlier studies have shown that 2-DG hinders the growth of several tumor cell types in vitro under hypoxic and normoxic conditions. In these studies, 2-DG causes these tumor cells to undergo cell death under hypoxic conditions only.[18]

One mechanism of 2-DG in targeting hypoxic cells is that it halts the process of glycolysis through inhibition of the catalytic enzyme hexokinase, which exerts control in the first step of the glycolytic pathway. Malignant cells express higher levels of hexokinase than normal, nonmalignant cells, suggesting that

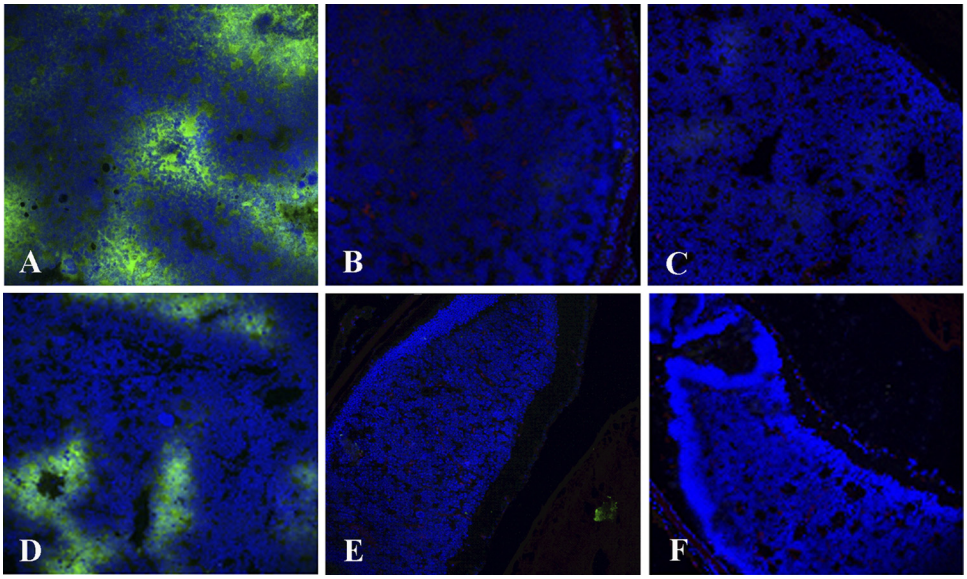

FIGURE 9. Hypoxia reduction after treatment with both treatments alone and in combination. (**A**) Saline-treated control group. (**B**) 250 mg/kg dose of 2-DG–treated eyes. (**C**) 500 mg/kg dose of 2-DG–treated eyes. (**D**) Carboplatin-treated eyes (31.25 μg/20 μL). (**E**) Carboplatin and 250 mg/kg dose of 2-DG–treated eyes. (**F**) Carboplatin and 500 mg/kg dose of 2-DG–treated eyes.

these cells require an elevated glycolytic metabolism to maintain the high demands of ATP essential for tumor growth.[37,38] We have previously suggested that the inhibition of glycolysis is a useful approach to overcome drug resistance associated with hypoxic cells in the tumor by killing these cells.[13] The present study corroborates this by showing that the glycolytic inhibitor 2-DG selectively targets the chemoresistant, hypoxic cells in $LH_{BETA}T_{AG}$ retinal tumors. The study further provides preliminary data to incorporate in the design of future clinical trials investigating the use of 2-DG in combination with other drugs to target slow-growing hypoxic cells in retinoblastoma.[18] Additionally, results from the series of studies using the glycolytic inhibitor 2-DG as adjuvant therapy to target the slowly proliferating cells in $LH_{BETA}T_{AG}$ retinal tumors may benefit other cancers and other chemoresistant malignancies of the central nervous system.

In conclusion, we further demonstrate that hypoxia is most prominent in advanced disease of $LH_{BETA}T_{AG}$ retinal tumors. This is the first study to show that periocular administration of 2-DG in the $LH_{BETA}T_{AG}$ retinoblastoma model has an effect on hypoxia and tumor burden. We have also corroborated previous results showing that the glycolytic inhibitor 2-DG selectively targets the chemoresistant, hypoxic cells. In addition, the use of periocular glycolytic inhibitors as adjuvant to chemotherapeutic agents, such as carboplatin, has the potential to enhance current retinoblastoma treatments.

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