

Mechanism of Retinoblastoma Tumor Cell Death after Focal Chemotherapy, Radiation, and Vascular Targeting Therapy in a Mouse Model

Maria-Elena Jockovich, Fernando Suarez, Armando Alegret, Yolanda Piña, Brandy Hayden, Colleen Cebulla, William Feuer, and Timothy G. Murray

PURPOSE. To evaluate the mechanism and timing of retinal tumor cell death in the LH_{BETA}T_{AG} mouse model of retinoblastoma after treatment with vascular targeting therapies and conventional therapies (focal chemotherapy and radiation).

METHODS. For vascular targeting therapy, 12- or 16-week-old mice were treated with a single subconjunctival injection of either anecortave acetate (300 µg) or combretastatin A4 (1.5 mg). Eyes were analyzed at 1 day and 1 week after treatment. Tumor cell death was evaluated using TUNEL assays or immunofluorescence analysis of activated caspase 3 to detect apoptosis. Histopathologic analysis was performed to identify areas of necrosis. For conventional therapy, LH_{BETA}T_{AG} mice were treated with six serial subconjunctival injections of focally delivered carboplatin chemotherapy (100 µg/delivery) or hyperfractionated external beam radiotherapy (EBRT; 15 Gy total dose). Cell death was analyzed by TUNEL assay.

RESULTS. The highest levels of apoptotic cell death were seen 1 day after treatment in all treatment groups compared with vehicle controls. At 1 week after treatment, apoptotic cell death remained significantly elevated in the EBRT and carboplatin groups, but not after vessel targeting therapy. No significant necrosis was detected by histology in tumors of treated or of control eyes.

CONCLUSIONS. Conventional therapies (focal carboplatin chemotherapy and EBRT) and vascular targeting agents significantly increase cell death through apoptosis, while not having a significant effect on necrosis in this murine model of retinoblastoma. These studies will aid in the optimization of delivery schemes of combined treatment modalities. (*Invest Ophthalmol Vis Sci.* 2007;48:5371-5376) DOI:10.1167/iovs.07-0708

Retinoblastoma, the most common intraocular tumor of childhood, occurs in approximately 1 in 15,000 to 1 in 16,600 live births in the United States.¹ Significant advances in treatment have resulted in 5-year survival rates in Europe and the United States of 90% and 98%, respectively.^{2,3}

Tumor control and globe conservation with preservation of sight have become the standard of care. Chemoreduction using

focal therapies have become a mainstay in the treatment of moderate and large tumors. However, concerns regarding secondary malignancies still exist. Novel therapeutic strategies are under investigation because of the morbidity and mortality associated with current therapies. These strategies include multimodality treatments using focally delivered chemotherapy and vascular targeting therapy.

Retinoblastoma tumors are characterized by aggressive growth, with continuing cellular proliferation occurring concomitantly along with cell death. Apoptosis, usually involved in normal development and tissue repair, sometimes occurs spontaneously in these malignant tumors, markedly retarding their growth.⁴⁻⁸ Extensive necrosis is often found and is associated with high-risk prognostic factors.⁹ Analysis of retinoblastoma cell death after treatment has been reported primarily in the study of cell lines.¹⁰⁻¹⁴ The induction of apoptosis is considered to be one of the principal mechanisms by which chemotherapy induces tumor regression.¹⁵ Treatment using carboplatin¹⁶ or radiation¹⁷ results in apoptotic cell death in retinoblastoma cell cultures. Although these treatments result in apoptotic cell death in vitro, studies of human retinoblastoma tumor samples after treatment have yielded inconclusive results. Studies of retinoblastoma patient specimens do not suggest a difference in apoptosis between treated and untreated groups.⁴ The lack of difference in apoptosis between these groups is likely attributed to the time lapse between treatment and analysis, which in most studies has been 6 weeks. In fact, cell death by apoptosis occurs within 1 week of treatment.¹⁸

Knowledge of the mechanism and timing of cell death after individual treatments is essential for combined modality therapy. However, it is not reasonable to analyze human retinoblastoma samples at specific time points, and cell lines do not fully replicate the intricate microenvironment of the intraocular tumor. Thus, animal modeling provides a way to optimize treatment strategies and to study tumor cell death after therapy.

Experiments using the LH_{BETA}T_{AG} mouse model of retinoblastoma suggest that combined treatment using carboplatin and EBRT¹⁹ or vascular targeting with anecortave acetate²⁰ enables a reduction in the effective dose of carboplatin, reducing associated toxicities.²⁰ Combined therapy is more challenging than single modality therapy in that treatment scheduling must be optimized to avoid adverse effects. Maximal treatment response from a combined therapeutic approach will require a highly coordinated dosing schedule that optimizes the timing of induced tumor cell death in response to either therapy.

In this study, we investigated the mechanism of cell death as a function of time after local vascular targeting agents (anecortave acetate or combretastatin A4) and conventional therapies (carboplatin chemotherapy or external beam radiotherapy [EBRT]) to best formulate an approach for the timing of combined therapeutic modalities in the future.

From the Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami School of Medicine, Miami, Florida.

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Corresponding author: Timothy G. Murray, Department of Ophthalmology, Bascom Palmer Eye Institute, P.O. Box 016880, Miami, FL 33101; tmurray@med.miami.edu.

METHODS

Animals

This study protocol was approved by the University of Miami Animal Care and Use Committee. All experiments in this study were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

The LH_{BETA}T_{AG} transgenic mouse model used in this study has been characterized previously.²¹ Presence of the SV40Tag was detected by PCR analysis of tail biopsies. To detect apoptosis during tumor progression, retinal tumors from LH_{BETA}T_{AG} mice of 4, 8, 12, and 16 weeks of age were analyzed ($n = 5$ per treatment group). LH_{BETA}T_{AG} mice typically develop microscopic tumors by age 4 weeks, 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.²²

Subconjunctival Injections and Drug Delivery

Injections were delivered with a 33-gauge needle inserted into the superotemporal subconjunctival space. LH_{BETA}T_{AG} mice of either 12 ($n = 5$ per group) or 16 ($n = 5$ per group) weeks of age received a single subconjunctival injection of anecortave acetate or combretastatin A 4P (CA4P). Drug doses that are known to result in the highest reduction of tumor burden were chosen for this study.^{20,23,24} Anecortave acetate (Alcon Pharmaceuticals, Forth Worth, TX) was delivered at a dose of 300 $\mu\text{g}/20 \mu\text{L}$; CA4P (OxiGene, Inc, Watertown, MA) was given at a dose of 1.5 mg/20 μL . Carboplatin was delivered to 10-week-old LH_{BETA}T_{AG} mice ($n = 6$ per group) by six serial, biweekly subconjunctival injections at a dose of 100 $\mu\text{g}/\text{delivery}$. Mice were humanely killed, and enucleations were performed at 1 day, 1 week, and 4 weeks after the termination of treatment.

Radiation

Ten-week-old LH_{BETA}T_{AG} mice ($n = 6$ per group) received EBRT in 120 cGy fractions delivered twice daily at a 6-hour interfraction interval for a total dose of 15 Gy (10 mV; Clinac 2100; Varian Medical Systems, Inc., Palo Alto, CA). Radiation dose and delivery schedule were chosen to obtain optimal response.²⁵ Mice were humanely killed 1 day, 1 week, and 4 weeks after termination of the last treatment. Eyes were fixed, paraffin embedded, and serially sectioned. Six sections per sample were obtained. The number of labeled cells in a $\times 400$ field was counted.

Detection of Apoptotic Cells

For the detection of apoptotic cells during tumor progression and after vessel targeting therapy, eyes were embedded in optimum cutting temperature compound, snap frozen in liquid nitrogen, and sectioned (8 μm). Sections were fixed with fresh 4% paraformaldehyde. Apoptosis detection was performed with a TUNEL based-kit (ApopTag Fluorescein In Situ Apoptosis Detection Kit; Chemicon, Temecula, CA) that detects apoptosis by adding digoxigenin nucleotides to the 3'-OH ends of double or single-stranded DNA. An anti-digoxigenin antibody conjugated with fluorescein is then added and followed by DAPI antifade (Chemicon) for immunofluorescence detection. Only cells that colocalized with DAPI fluorescence were counted as positive cells. Apoptotic cell death was also detected with a rabbit antiactivated caspase 3 (AC3) antibody (1:100; Chemicon). Goat anti-rabbit fluorescent dye (Alexa Fluor 568; 1:500; Invitrogen, Carlsbad, CA) was used as a secondary antibody. Vascular endothelial cells were detected with fluorescent dye-conjugated lectin (*Bandera simplicifolia*; Alexa Fluor 568; 1:1000; Invitrogen). The ratio of endothelial cell death to apoptotic cell death was given as the number of apoptotic cells (as determined with AC3) that colocalized with lectin to the total number of apoptotic cells.

Serial cross-sections of eyes containing tumors were examined for the presence of the different markers with an upright fluorescence microscope (BX51; Olympus America Inc., Melville, NY). All images

were digitally acquired and recompiled (Photoshop CS; Adobe, San Jose, CA). Sections were viewed at $\times 40$ magnification.

For the detection of apoptosis after carboplatin treatment or EBRT, eyes were analyzed at 1 day, 1 week, and 4 weeks after termination of the last treatment. Eyes were fixed with 10% formalin and embedded in paraffin. In situ detection of apoptotic cells was conducted using the anti-BRDU antibody and a detection kit (TACS; R&D Systems, Minneapolis, MN). Labeled cells in $\times 400$ fields were counted. At least three fields were counted and averaged.

Statistical Methods

Unless otherwise specified, data were analyzed according to analysis of variance (ANOVA). Post hoc tests were performed by the least significant difference test.

RESULTS

Tumor Cell Death during Tumor Progression

To assess cell death during tumor development, retinal tumors from LH_{BETA}T_{AG} mice of 4, 8, 12, and 16 weeks of age were analyzed for apoptosis using both the TUNEL assay and a marker for AC3. In LH_{BETA}T_{AG} mice, cell death was not detected in small tumors (4–8 weeks of age). In larger tumors, low levels of apoptosis were detected (2–3 per high-power field; $\times 400$; Fig. 1). The highest numbers of apoptotic cells were seen in retinal tumors from 12-week-old mice. The levels of apoptosis detected by the TUNEL assay at 12 weeks of age appeared higher than those detected by AC3. However, this difference was not statistically significant ($P = 0.133$). Sixteen-week-old mice had a mean number of apoptotic cells that was significantly lower than the mean number of apoptotic cells in 12-week-old mice ($P = 0.002$, TUNEL; $P = 0.052$, AC3). Necrosis was not detected by histology in these eyes (data not shown).

Tumor Cell Death after Vessel Targeting Therapy

To assess cell death after vessel targeting therapy, 16-week-old LH_{BETA}T_{AG} mice ($n = 5$ per group) were treated with a single subconjunctival injection of either anecortave acetate (300 μg) or CA4P (1.5 mg). Tumors were analyzed for apoptosis or necrosis 1 day or 1 week after treatment. Both TUNEL assay and AC3 immunohistochemistry yielded the same results. There was significantly higher apoptotic cell death 1 day after vessel targeting therapy with either agent than the untreated age-matched controls ($P < 0.001$; both, Fig. 2). The data further suggest a differential induction of tumor cell death relative to time of treatment and vascular targeting agent. The highest amount of apoptotic cell death was detected with both treatments 1 day after injection. The number of apoptotic cells in these advanced tumors decreased significantly by 1 week after treatment ($P = 0.002$, anecortave acetate; $P < 0.001$, CA4P). Statistical analysis of AC3 data suggests that the amount of apoptotic cell death varies with treatment. The two drugs/two time points and controls were compared in a one-way ANOVA. The average count of all fields was examined for each animal, and the average apoptotic cell count of all fields was examined for each animal. Average numbers of apoptotic cells were as follows: no treatment, 1.8; 1 day anecortave acetate, 11.3; 1 week anecortave acetate, 3.5; 1 day CA4P, 25.5; 1 week CA4P, 3.5. The differences in apoptotic cell count between treatment groups 1 day after injection, between treated groups 1 day after injection relative to untreated controls, and between 1 day and one 1 week time points after treatment with each drug were highly significant ($P < 0.001$). There was no significant difference between the two 1-week posttreatment groups (Fig. 2).

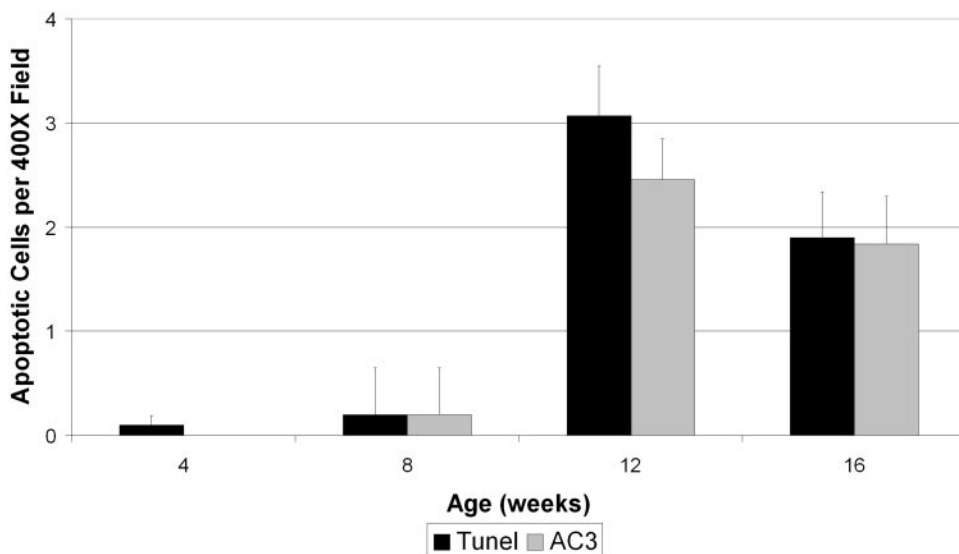


FIGURE 1. Natural history of tumor cell death. Bars represent number of apoptotic cells per high-power field (HPF; $\times 400$) marked with TUNEL (■), or activated caspase 3 (■). Error bars represent SD from the mean ($n = 5$). The difference in mean apoptotic numbers measured by TUNEL and activated caspase 3 is not statistically significant ($P = 0.13$).

Endothelial Cell Death after Vessel-Targeting Therapy

To assess the amount of endothelial cell death after vessel-targeting therapy, LH_{BETA}T_{AG} mice were treated at 12 or 16 weeks of age with anecortave acetate or CA4P. Eyes were analyzed by immunohistochemical analysis for AC3 and lectin at 1 day and 1 week after treatment. Our data show that only approximately 50% of total apoptotic cells after treatment with anecortave acetate or CA4P are endothelial cells (Fig. 3). Endothelial to total apoptotic cell ratio is significantly higher in treated animals than in untreated littermate controls at 12 weeks ($P = 0.004$, two-sample *t*-test) and 16 weeks ($P = 0.005$, two-sample *t*-test). The mean ratio of endothelial cell death to total cell death in the 12-week treatment groups was 0.560 ± 0.26 (\pm SE); at 16 weeks of age, the mean was 0.453 ± 0.21 (\pm SE). Further analysis by three-factor ANOVA showed that there was a significant difference ($P = 0.003$) in endothelial cell death between the 12-week and the 16-week age groups but not between treatment agents ($P = 0.76$) or days after treatment ($P = 0.87$). These results suggest that vascular targeting therapy is more effective in promoting endothelial cell apoptosis when mice are treated at 12 weeks of age than when

they are treated at 16 weeks of age. This difference appeared to be more evident when eyes were analyzed 1 day after anecortave acetate treatment. Necrosis was not detected in these eyes after vessel targeting therapy with either agent at either time point (not shown).

Tumor Cell Death in Response to Conventional Therapies

To assess cell death after either radiotherapy or carboplatin chemotherapy, 10-week-old mice received carboplatin or EBRT as described in Methods. Eyes were analyzed 1 day, 1 week, and 4 weeks after treatment completion (Fig. 4). At day 1, eyes treated with 100 μ g carboplatin chemotherapy exhibited a 10.6-fold increase in the percentage of apoptotic cells over control eyes. This increased slightly at 1 week to 12.1-fold. By 4 weeks after treatment, the levels of apoptotic cell death were down to those seen in untreated controls. Eyes treated with EBRT at a dose of 15 Gy also exhibited an increase in apoptotic cell death compared with the control. There was a 10.3% increase at day 1, a 4.4% increase at 1 week, and no significant difference at 4 weeks relative to the control samples. Control eyes in this model revealed approximately a 1% incidence of

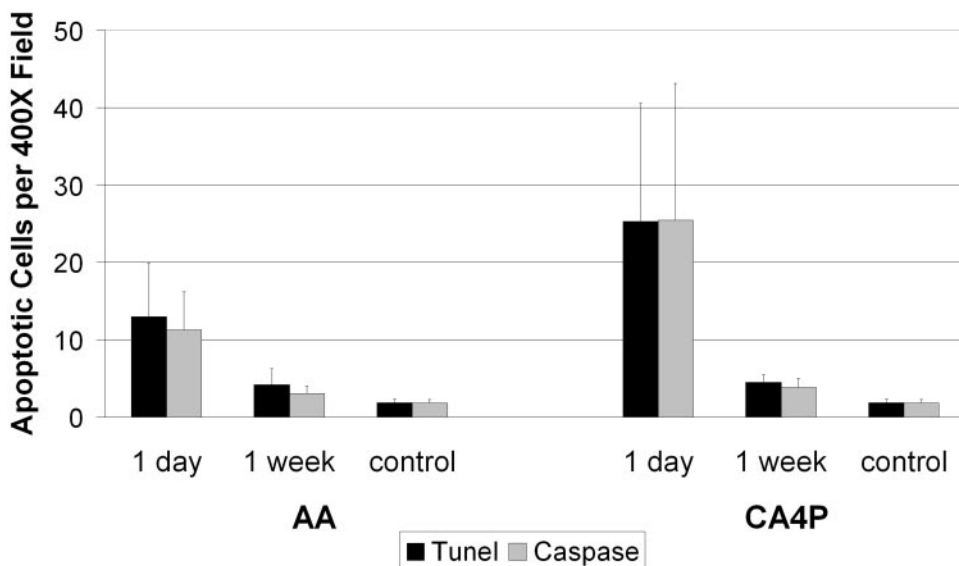


FIGURE 2. Tumor cell death after vessel targeting therapy. LH_{BETA}T_{AG} mice of 16 weeks of age were treated with single subconjunctival injection of either anecortave acetate (300 μ g) or CA4P (1.5 mg). Bars represent number of apoptotic cells per high-power field (HPF; $\times 400$) marked with TUNEL (■) or activated caspase 3 (■). Error bars represent SD from the mean ($n = 5$).

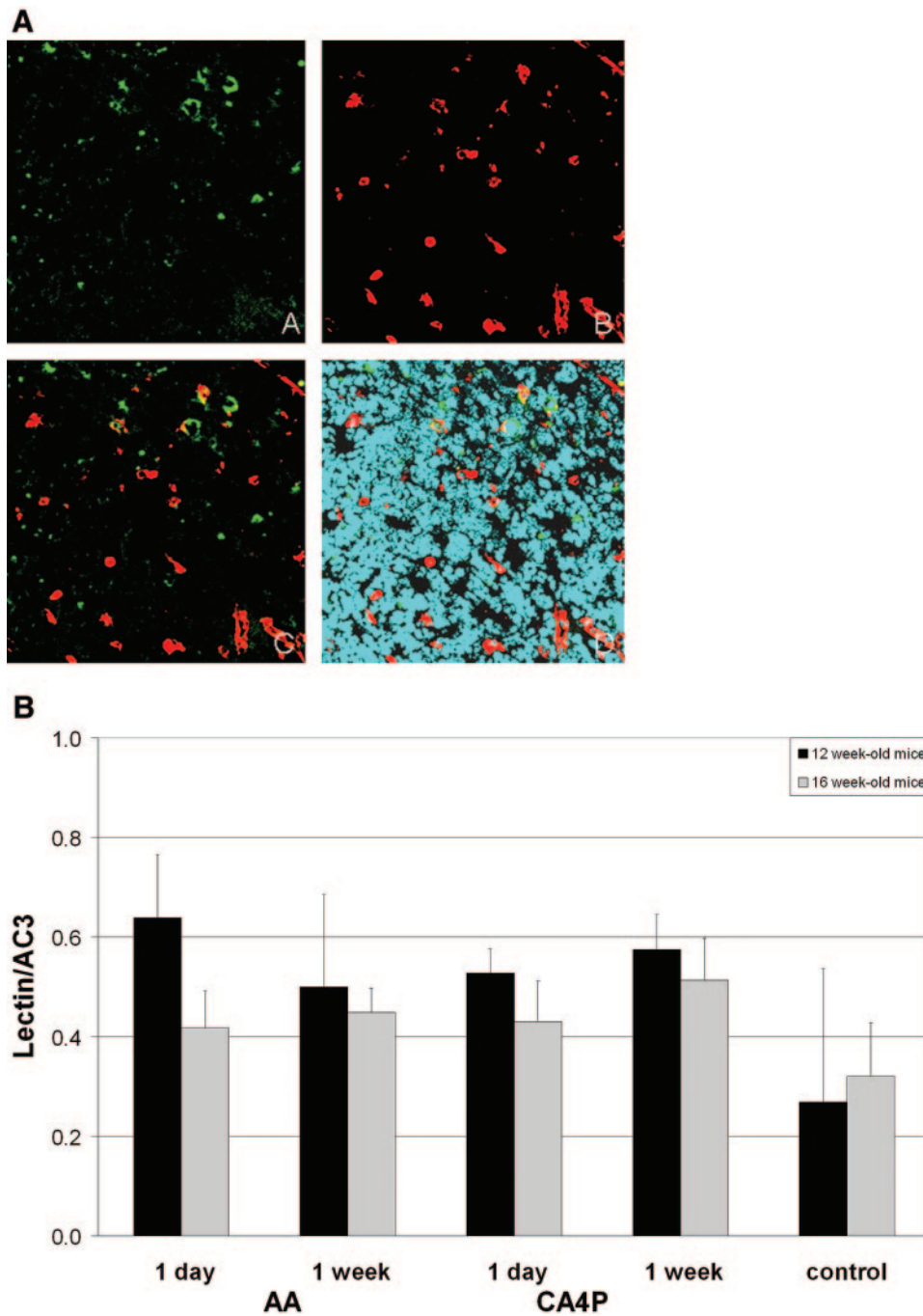


FIGURE 3. Percentage of apoptotic endothelial cells relative to total apoptotic cells after vessel targeting therapy in mice of 12 weeks and 16 weeks of age. (A) Representative micrograph illustrating apoptotic cells and endothelial cells 1 day after treatment with anecortave acetate. Apoptotic cells stained with activated caspase 3 (A), endothelial cells stained with lectin (B), colocalization (C), and DAPI nuclear stain (D). (B) Bars represent percentage of apoptotic cells that colocalized with endothelial cells to total apoptotic cells per high-power field in animals treated at 12 weeks (■) and 16 weeks (▒) of age. Error bars represent SD from the mean ($n = 5$).

cells in apoptosis throughout the time points examined. The percentage of cells in apoptosis ranged from 0.6% at 1 week to 1.8% at 4 weeks. No statistical difference was noted at any time point within these control eyes. These results show that at 1 day and 1 week, both the carboplatin and the EBRT groups have a significantly greater incidence of apoptotic cell death than the control ($P < 0.001$ and $P < 0.02$, respectively). At 1 week after treatment, carboplatin-treated tumors had a significantly greater percentage of cells in apoptosis than EBRT-treated tumors ($P = 0.007$) and untreated controls ($P = 0.001$). At this time point, EBRT-treated tumors also had significantly higher levels of apoptosis than untreated controls ($P = 0.037$). The levels of apoptosis had decreased by 4 weeks after both treatments.

DISCUSSION

Novel therapeutic strategies for the treatment of retinoblastoma optimally involve targeted timed multimodality approaches for tumor control. Optimal combined therapies require an understanding of the pharmacokinetics of each individual drug and the mechanism and timing of cell death after individual treatments. In this study, the timing and mechanism of retinal tumor cell death were investigated after treatment with commonly used therapeutics (carboplatin and radiation) and the novel antiangiogenic and angiostatic therapeutic strategies under investigation. Data from this study suggest that apoptosis is the mechanism for cell death in the LH_{BETA}T_{AG} mouse model of retinoblastoma undergoing radiotherapy, focal

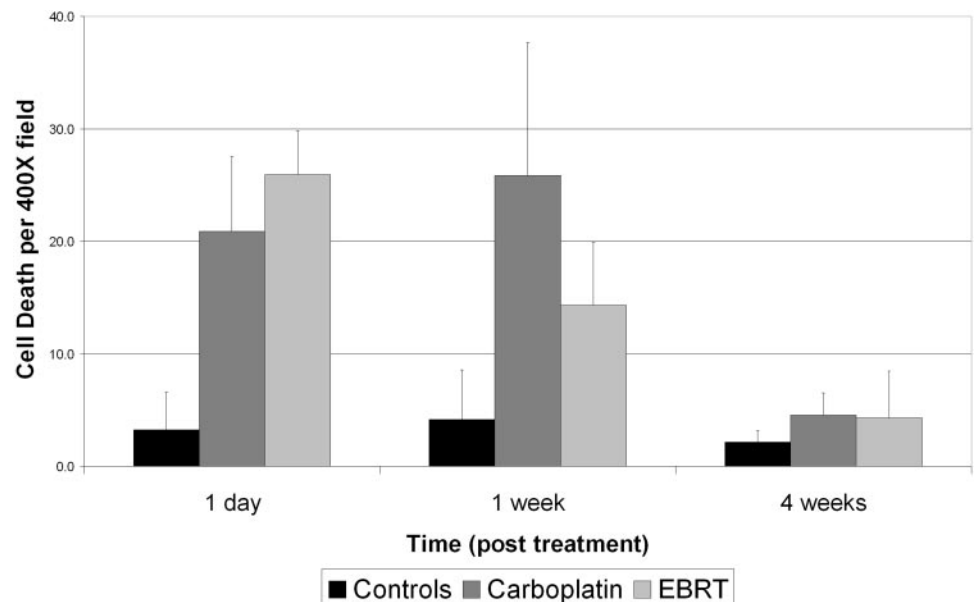


FIGURE 4. Differential apoptosis after chemotherapy and EBRT. $LH_{BETA}T_{AG}$ mice of 10 weeks of age were treated with six bi-weekly subconjunctival injections of carboplatin (100 μ g/delivery) or EBRT (120 cGy fractions for a total dose of 15 Gy). Bars represent percentage of apoptotic cells in untreated controls, (■), after treatment with carboplatin (■), or EBRT (■). Error bars represent SD from the mean ($n = 6$).

chemotherapy, or vessel-targeting therapy. Differential induction of apoptosis is noted after treatment with early enhancement associated with radiotherapy and vessel targeting therapy and late enhancement with chemotherapy.

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. We demonstrated that two different vessel-targeting agents (combretastatin A4 and anecortave acetate) effectively reduce tumor burden in the $LH_{BETA}T_{AG}$ model of retinoblastoma.^{20,23,26} Our previous work also found that when anecortave acetate is used together with carboplatin, the dose and delivery schedule must be optimized to avoid adverse effects.²⁰ Data from the present study suggest that both agents induce rapid caspase-dependent apoptotic cell death within 1 day of treatment.

A higher percentage of endothelial cell death to total cell death was detected in mice treated at 12 weeks of age than in mice treated at 16 weeks of age. A plausible explanation for this finding is that younger mice have a higher percentage of angiogenic vasculature than older mice. We have recently characterized blood vessel maturation in the $LH_{BETA}T_{AG}$ mouse model of retinoblastoma.²² Angiogenesis in developing retinal tumors was detected in the early stages of tumor development and increased with age, decreasing slightly in advanced disease. On the other hand, tumor vessel maturation does not occur until advanced disease develops at 12 to 16 weeks of age; the amount of pericyte-committed vasculature increases with age. Endothelial cells in newly formed vessels require growth factors for survival; in their absence, such as after antiangiogenic treatment, the endothelial cells undergo apoptosis and regress.²⁷ Mature vessels are stabilized by pericytes and are no longer dependent on angiogenic stimuli, thus, they may be resistant to antiangiogenic treatment. We have reported that treatment with vessel-targeting agents CA4P and anecortave acetate in advanced disease, though reducing total numbers of endothelial cells, did not effectively target mature vasculature.²² 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.

Antiangiogenic and cytotoxic chemotherapy potentially yield maximal effects when combined because different cells

in the tumor mass are targeted cancer cells and endothelial cells.²⁸ Targeting vasculature, however, may compromise the delivery of chemotherapy to the tumor and may antagonize the effect of the combined therapy.²⁹ Studies have shown that endothelial cell apoptosis precedes tumor cell death in many solid tumors.³⁰ The premise behind the use of antiangiogenic therapy for solid tumors is that though it kills endothelial cells that feed the tumor, causing tumor cell death, these capillaries are also responsible for delivering chemotherapeutic drugs to the tumor. Thus, vessel targeting may inhibit coincident drug delivery. We have previously shown that the greatest reduction of tumor burden is achieved when anecortave acetate treatment follows a cycle of six carboplatin injections; if anecortave acetate is given during the carboplatin cycle, then the synergistic effect of the combination of the two drugs is lost.²⁰ Based on the data from the present study and our previous results, we believe that an ideal dosing regimen would be six cycles of carboplatin chemotherapy followed 1 week later by a single injection of anecortave acetate. This delivery scheme would presumably increase tumor cell death while minimizing toxic adverse effects associated with high doses of chemotherapy.

The vessel-targeting agents used have different mechanisms of action: anecortave acetate is an angiostatic cortisone that prevents new endothelial blood vessel formation by inhibiting growth factors required for endothelial cell survival.³¹ CA4P induces endothelial cell death by arresting cells in mitosis.³² The ensuing vessel collapse results in a rapid ischemic necrotic cell death in surrounding tumor cells. Interestingly, we detected the same type of cell death after treatment with either drug. No signs of necrosis were detected in these tumors.

In conclusion, this study demonstrates that apoptosis is a mechanism for cell death in the $LH_{BETA}T_{AG}$ mouse model of retinoblastoma undergoing radiotherapy, subconjunctival chemotherapy, or vessel-targeting therapy. Further, we have shown that there is a differential timing of the induction of apoptosis after different treatment modalities. This differential timing of induction of apoptosis may account for synergistic therapeutic interactions noted during combined treatment and may have implications in combined modality therapies for retinoblastoma.

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