External Beam Radiation “Salvage” Therapy in Transgenic Murine Retinoblastoma

Objective: To determine the efficacy of low-dose “salvage” external beam radiation therapy (EBRT) following failed subconjunctival carboplatin chemotherapy in a murine model of heritable retinoblastoma.

Methods: Eighty-four eyes from 8-week-old, simian virus 40, T-antigen–positive mice were treated with 6 serial subconjunctival carboplatin injections (100 µg/25 µL). At 12 weeks of age, 64 eyes received EBRT for a total dose of 480 (4.8 Gy), 1200 (12.0 Gy), 1560 (15.6 Gy), or 3000 (30.0 Gy) rad. Twenty eyes received no additional therapy following subconjunctival carboplatin injections. Ten eyes received a total dose EBRT of only 3000 rad. Eight eyes received subconjunctival injections of only an isotonic sodium chloride solution. Ten eyes served as untreated controls.

Main Outcome Measures: Eyes were enucleated at 20 weeks to assess the presence of tumor on histopathological examination.

Results: Salvage therapy using low-dose EBRT was able to reestablish tumor control in a dose-dependent manner. Increasing the EBRT dose to 3000 rad resulted in 100% tumor control. The dose-dependent curves were significantly different between the treatment groups—EBRT alone vs salvage EBRT after receiving subconjunctival carboplatin injections ($P < .001$).

Conclusion: Low-dose hyperfractionated salvage EBRT following failed primary subconjunctival carboplatin chemotherapy is efficacious in the treatment of retinoblastoma in this animal model.

Clinical Relevance: Salvage EBRT using a reduced total radiation dose could be associated with a radiation-related treatment enhancement in pediatric retinoblastoma.

From the Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami, Miami, Fla (Drs Sobrin, Murray, Scott, Wu, and Markoe, Mss Hayden and Cicciarelli, and Messrs Hernandez and Feuer); and the Department of Ophthalmology, University of California, San Francisco (Ms Fulton and Dr O’Brien). The authors have no relevant financial interest in this article.

The early diagnosis and treatment of intraocular retinoblastoma have contributed to a marked improvement in patient survival.1,2 Treatment strategies for retinoblastoma have been evolving significantly over the past several years. Current treatment modalities include enucleation, external beam radiation therapy (EBRT), scleral plaque radiotherapy, transpupillary thermoablation, cryotherapy, laser ablation, and chemotherapy.3-6 External beam radiation therapy has played a prominent role in the treatment of advanced stages of retinoblastoma, especially in children with a germ-line Rb mutation predisposing them to bilateral disease.7,8 Although efficacious, ionizing radiation exposure has been associated with severe complications such as facial deformities, cataract, radiation-induced retinopathy, radiation-induced vasculopathy, optic neuropathy, and an enhanced risk of secondary malignant tumors.9-19 In recent years, systemic chemotherapy has been increasingly used in the primary treatment of moderate to large retinoblastoma tumors (Reese-Ellsworth stages III-V).20-27 Chemotherapy has been demonstrated to improve the rates of ocular salvage in selected patients and has decreased the need for EBRT or postponed the use of EBRT in many cases.

Several clinical trials have documented the efficacy of systemic carboplatin therapy in the treatment of multiple neoplasms in children.28,29 Although this agent has played a pivotal role in virtually all chemotherapeutic regimens proposed for the treatment of retinoblastoma, owing to its low toxic effect profile compared with other chemotherapeutic agents, concerns regarding significant morbidity and potential mortality caused by drug-related toxicity still exist.30-33 As a result, the use of focal chemotherapy has been recently advocated. This treatment modality should include the benefits associated with chemoreductive treatment and would conceivably spare patients the associated
adverse toxic effects and mutagenic potential of systemic delivery. Our laboratory has documented the efficacy of focal carboplatin chemotherapy, provided intravitreally and subconjunctivally, in the treatment of a transgenic murine model of retinoblastoma. Measurement of intraocular carboplatin concentration after peribulbar administration in nontumor-bearing primates has shown dramatically higher concentrations of carboplatin in the vitreous and aqueous humor than in control animals receiving intravenous administration of the drug. Abramson et al have reported significant efficacy in control of vitreous seeding and primary retinal tumor in a phase I/II clinical study of subconjunctival carboplatin administration for intraocular retinoblastoma.

This focal approach has enabled researchers to concentrate on methods of combining chemotherapy with other local treatment modalities. Combined modality therapy, especially if acting synergistically, may allow a reduction in treatment dose, minimizing dose-dependent complications. Several investigators have evaluated the application of chemoradiative therapy with aggressive local treatment to eliminate the need for EBRT. Others have evaluated focal plaque radiotherapy, as a primary and secondary treatment, in the hopes of decreasing orbital and central nervous system radiation exposure. However, EBRT is often needed as “salvage” therapy after failure of systemic chemoreduction to eradicate vitreous or subretinal seeding in the management of large retinoblastoma tumors. The aim of this study is to evaluate the efficacy of low-dose salvage EBRT following failed subconjunctival carboplatin chemotherapy in a murine model of spontaneous heritable retinoblastoma.

METHODS

This study protocol was approved by the University of Miami School of Medicine Animal Care and Use Review Board, Miami, Fla. All experiments in this study were conducted in accord with the Association for Research in Vision and Ophthalmology guidelines for the use of animals in ophthalmologic and vision research.

One hundred twelve eyes from simian virus 40, large T-antigen transgene-bearing mice were treated, as described below, beginning at 8 weeks of age. The transgenic mouse model used in this study has been characterized previously. Briefly, a highly expressed murine oncogenic transgene drives bilateral retinal tumor development by using a retinal-specific promoter sequence to direct the expression of simian virus 40 T antigen, resulting in ocular tumor growth. Transgenic animals were identified through polymerase chain reaction analysis of tail DNA. Samples positive and negative for the transgene were detected by visualizing ethidium bromide–stained agarose gels. Transgene-positive animals develop bilateral, heritable retinoblastoma that resembles human retinoblastoma including similar histopathological, immunocytochemistry, and behavioral growth patterns. Pathological evidence of tumor is noted by the age of 4 weeks, a small intraocular tumor (corresponding to Reese-Ellsworth stage I) present at 5 weeks, a moderate to large intraocular tumor (corresponding to Reese-Ellsworth stages III and IV) is noted at 10 weeks, and the tumor fills the globe by 16 weeks. Tumors in this animal model are typically small at 5 weeks, appearing with intraretinal involvement only (occupying <1% of retinal area and correspondingly less ocular volume), and at 10 weeks the tumor is moderate in size (occupying approximately 20%-25% of the retinal area and 10%-25% of the ocular volume).

SUBCONJUNCTIVAL CARBOPlatin INjECTIONS

Eighty-four eyes from 8 week-old, simian virus 40, T-antigen–positive mice were treated with subconjunctival carboplatin (Paraplatin; Bristol-Myers Squibb, Princeton, NJ) injections (100 µg/25 µL) administered twice per week for a total of 6 injections. Animals were anesthetized with a combination of intraperitoneal ketamine hydrochloride and xylazine hydrochloride. Carboplatin injections were provided with a 33-gauge needle inserted into the nasal and superior subconjunctival spaces. A microvolume delivery pump was used to ensure accurate and reproducible delivery of a 23-µL volume. Twenty eyes received no additional therapy following subconjunctival carboplatin injections. Ten eyes of litter-matched animals served as untreated controls. Eight eyes of litter-matched animals received 25-µL subconjunctival injections of balanced salt solution to provide a positive placebo control. Following each injection, all animals underwent external ophthalmologic and fundus examination to ensure no perforation of the globe had occurred.

ORBITAL EBRT

Mouse eyes were treated, as per treatment groups described below, with fractionated EBRT using a 10-mV x-ray machine (CLINAC-2100: Varian Medical Systems, Charlottesville, Va) (Figure 1). Unanesthetized animals were briefly immobilized in specially constructed cages and shielded to minimize irradiation to midline nonocular structures. Treatment ports were confirmed and radiation was delivered at 324 rad/min (3.24 Gy/min) in a 7.0 × 7.0-cm field. All radiation treatments were delivered twice per day in 120-rad (1.2-Gy) fractions from 6 to 8 hours apart. Ten eyes received only EBRT at a total dose of 3000 rad (30.0 Gy) to provide a positive placebo control.

COMBINED SUBCONJUNCTIVAL INJECTIONS AND EBRT

At 12 weeks of age, 64 eyes from the subconjunctival carboplatin–treated mice received EBRT for a total dose of 480 (4.8 Gy) (n=12), 1200 (12.0 Gy) (n=10), 1560 (15.6 Gy) (n=26), or 3000 (30.0 Gy) rad (n=16).

HISTOPATHOLOGICAL EXAMINATION

At 20 weeks of age, all animals were killed with an overdose of combined ketamine and xylazine. Both eyes were enucleated and immediately immersion fixed in a 10% formalin solution.
The eyes were sectioned serially and stained with hematoxylin-eosin. Light microscopic examination was performed on all histopathological sections in a masked fashion. Eyes were graded positive for tumor development if any histopathological evidence of tumor was present. Eyes were also evaluated for evidence of toxic effects to the cornea, lens, retina, or sclera.

STATISTICAL ANALYSIS

To determine the dose-response relationships among the different treatment doses, data were subjected to Probit statistical analysis. Probit is a standard method of dose response. Probit regression allows the assessment of the effects of drug concentration and treatment group for dose-response data in which the assumptions of standard linear regression are not met. Total radiation dose was entered as a linear predictor in a maximum likelihood Probit regression model. The model was assessed for goodness-of-fit to ensure that assumptions of the Probit model were not violated. The radiation enhancement ratio was calculated by dividing the tumor control dose for 50% of eyes (TCD50) for 3000-rad EBRT alone by the TCD50 for 3000-rad salvage EBRT after subconjunctival carboplatin administration.

RESULTS

Histopathological examination at age 20 weeks revealed that all untreated eyes (n=10) and isotonic sodium chloride solution–treated eyes (n=8) exhibited multiple large intraocular tumors (Figure 2). Tumor control was achieved in only 20% of the mice treated with 100 µg of subconjunctival carboplatin alone.

COMBINED SUBCONJUNCTIVAL INJECTIONS AND EBRT

Salvage therapy using low-dose EBRT was able to reestablish tumor control in a dose-dependent manner. Tumor control was not achieved in any of the eyes treated with a total dose of 480 rad after subconjunctival carboplatin therapy. Eradication of tumor was seen in 30% of eyes treated with subconjunctival carboplatin followed by EBRT for a total dose of 1200 rad (Figure 3). Aver-
age tumor size in this group treated with carboplatin and 1200-rad (12.0-Gy) EBRT was smaller than the average tumor size in the group treated with carboplatin alone. Sixty-five percent tumor control was achieved with combined treatment of carboplatin and 1560-rad (15.6-Gy) total dose EBRT (Figure 4) and complete tumor eradication occurred in eyes treated with carboplatin and EBRT at a total dose of 3000 (30.0 Gy) rad (Figure 5). In comparison, EBRT alone at 120-rad (1.2-Gy) fractions twice per day failed to eradicate tumor in 70% of eyes treated with a total dose of 3000 rad (Figure 5). There was no histopathological evidence of ocular toxic effects for any of the treated eyes.

PROBIT REGRESSION ANALYSIS

Probit regression analysis demonstrated that the dose-dependent curves were significantly different between the treatment groups (EBRT alone vs salvage EBRT after subconjunctival carboplatin injections) \((P<.001)\) (Figure 6). The regression coefficient for EBRT salvage therapy was 2.80 with a 1.59 to 4.01 ninety-five percent confidence interval. The radiation enhancement ratio was 2.39 with 100-µg subconjunctival carboplatin injections.

COMMENT

Earlier detection and refinement of conservative methods of treatment of retinoblastoma have led to fewer primary enucleations, high globe-conservation rates, and better prognosis for visual outcome. Life expectancy following treatment is excellent, but survivors with genetic predisposition face an increased risk of subsequent cancers. Radiotherapy further increases the risk for a second primary tumor, with a cumulative incidence of 35% during a 30-year follow-up in radiation-treated patients, compared with 5.8% in those not receiving EBRT. This has led to the search for alternate treatment modalities.

Several groups have demonstrated the efficacy of initial systemic chemotherapy in reducing tumor size (chemoreduction). Systemic chemotherapy also enhances the effectiveness of other treatment modalities and may reduce the possibility of metastatic dissemination. Disadvantages include serious toxic adverse effects, potential mutagenesis, and increased risk for development of other malignant neoplasms. Furthermore, though effective in initial tumor reduction, this treatment modality alone is unable to achieve complete tumor control and, therefore, often requires adjuvant local therapy. Chemoreduction combined with local therapies including laser hyperthermia and cryoablative therapy have been used with much success in recent years. Despite these advances in tumor control, recent studies indicate that even the combination protocols are often insufficient to eradicate vitreous or subretinal seeding, requiring salvage EBRT to establish more complete tumor control. The reasons for the failure of this treatment modality to eliminate vitreous and subretinal seeding include insufficient penetration of the intraretinal and intravitreal spaces by the systemically administered chemotherapeutic drug, dose limitations, and rapid renal clearance of the drug.
Local delivery of carboplatin therapy may be more beneficial than intravenous delivery because it is associated with increased drug penetration of ocular structures and tumor control in animal models. Previous studies in our laboratory have demonstrated the ability of carboplatin administered intravitreally, subconjunctivally, and iontophoretically to completely eliminate tumor in the treatment of murine retinoblastoma.\textsuperscript{34-36,42,49} Peribulbar carboplatin administration in nontumor-bearing primates produces dramatically higher concentrations in the vitreous and aqueous humor compared with intravenous administration of the drug.\textsuperscript{38} Recent experiments in our laboratory on the pharmacokinetics of local vs systemic carboplatin administration in the rabbit eye show that intraretinal and intravitreal drug concentrations following subconjunctival injection or iontophoretic provision are significantly higher than those achieved with systemic administration (B.C.H., T.G.M., E.H., and Monika Viogt, MD, Peter Milne, PhD, Martina Kralinger, MD, and Jean-Marie Parel, PhD, unpublished data, January 2001). One clinical trial of subconjunctival carboplatin administration for intraocular retinoblastoma has also shown it to be efficacious in children.\textsuperscript{39} Direct subconjunctival delivery of carboplatin chemotherapy may be associated with decreased toxic effects and considered safe when administered at optimum concentrations and dose schedules.\textsuperscript{34}

Use of local chemotherapeutic drug delivery as part of a combined modality protocol further limits toxic effects. Platinum compounds may have a radiosensitizing effect on hypoxic tumors, such as retinoblastoma, which could contribute to the potential efficacy of combined modality therapy with carboplatin and low-dose irradiation.\textsuperscript{49} Our current study shows that low-dose hyperfractionated salvage EBRT following failed primary carboplatin chemotherapy is efficacious in the treatment of retinoblastoma in this transgenic animal model. The total dose provided with EBRT may be reduced by 58%, while maintaining local tumor control.

In this murine retinoblastoma model, the TCD$_{50}$ has recently been found to be 3370 rad (33.7 Gy) for those animals administered radiation therapy in 120-rad (1.2-Gy) fractions twice per day.\textsuperscript{66} In our study, the TCD$_{50}$ for those animals administered salvage EBRT after failed carboplatin therapy decreased to 1410 rad (14.1 Gy). The radiation enhancement ratio, which describes the potential enhancement of combined therapy of carboplatin and radiation in this tumor model, is calculated to be 2.39. The TCD$_{50}$ for EBRT used in the calculation was taken from the Hayden et al\textsuperscript{66} study because of its larger sample size (n=42). In this prior study, the mice were killed at...
16 weeks instead of 20 weeks. Therefore, the radiation enhancement ratio may actually be underestimated as earlier age at death in the EBRT-only group may mask subsequent tumor growth.

The degree of enhancement described by the radiation enhancement ratio suggests the ability to decrease the dose of both carboplatin and radiation therapy without any compromise in tumor control. This approach may allow for a decrease in the morbidity associated with radiation therapy or chemotherapy. If used as a primary modality for the treatment of retinoblastoma, the potentiation between carboplatin and radiation therapy may result in a more complete initial response, leading to fewer recurrences. If used as secondary treatment after failure of chemotherapy, EBRT may be used at lower doses than previously believed necessary to achieve tumor control, minimizing the adverse effects of radiation therapy and the long-term secondary cancer risks of this treatment. Because carboplatin is a known radiation sensitizer, however, it could increase normal tissue complications or increase the risk of secondary cancers in combination with EBRT.

Several considerations remain before applying the information gained in this study to childhood retinoblastoma. First, the transgenic model may differ from human retinoblastoma in its response to treatment. For example, radiation-related complications, including optic neuropathy and retinal vasculopathy, are rarely seen in the mouse eye. Second, although subconjunctival carboplatin therapy avoids problems of systemic toxic effects and showed no signs of toxic effects in mice, it is difficult to predict the effect of carboplatin therapy on the final visual acuity in humans. The potential for long-term ocular toxic effects from combination local carboplatin therapy and EBRT is unknown.

External beam radiation therapy using a reduced total radiation dose as salvage therapy could be associated with a radiation-related treatment enhancement, permitting tumor control, while minimizing treatment-related toxic effects in pediatric retinoblastoma.

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Corresponding author: Timothy G. Murray, MD, Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami, PO Box 016880, Miami, FL 33101 (e-mail: tmurray@med.miami.edu).

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