Local Carboplatin Therapy in Transgenic Murine Retinoblastoma

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Purpose. To determine the efficacy and toxicity associated with intraocular delivery of carboplatin in the treatment of murine transgenic hereditary retinoblastoma.

Methods. Forty-eight transgenic BLH-SV40 Tag retinoblastoma mice were administered five intravitreal injections of carboplatin in one eye. After 12 weeks, the eyes were examined histopathologically to evaluate tumor burden. Twelve rabbits were administered intravitreal injections of carboplatin in one eye, after which they underwent serial electroretinography. All experimental and control eyes were obtained for histopathology and electron microscopy.

Results. A dose-dependent inhibition of intraocular tumor growth by carboplatin was observed in transgenic retinoblastoma mice. Tumor development was inhibited in 50% of the mouse eyes at doses of 1.4 μg. In rabbits, retinal toxicity resulted when intravitreal injections of carboplatin were administered at doses of 10 μg or higher.


Retinoblastoma is the most common primary intraocular malignancy in children.1 The survival rate in developed countries has increased dramatically in recent years through early diagnosis and appropriate treatment with enucleation, external beam radiotherapy, episcleral plaque radiotherapy, cryotherapy, and laser photoablation.1 Although external beam radiotherapy plays a prominent role in treatment, especially in bilateral cases, it is associated with substantial morbidity, including facial deformities and second primary tumors in the field of radiation.1 To avoid these radiation-related complications, some physicians have advocated systemic chemotherapy in lieu of external beam radiotherapy as a primary treatment modality in some intraocular retinoblastomas.2–12

Systemic chemotherapy has been used in patients with retinoblastoma if there is orbital extension or distant metastasis.13–16 Cyclophosphamide and vincristine are among the more effective agents but are associated with substantial toxicity.17–19 Platinum compounds, such as cisplatin and carboplatin, are less toxic and are used increasingly in pediatric oncology.13–16,18 Carboplatin is used in most regimens for intraocular retinoblastoma.10–12

Despite the relatively favorable toxicity profile for carboplatin, significant side effects have been observed, including myelosuppression, nephrotoxicity, ototoxicity, sepsis, second tumors, and death.15,19–22 Children with bilateral retinoblastoma have a strong genetic cancer diathesis and may be at even greater risk for mutagenesis and second tumors.23–25 Although external beam radiotherapy clearly contributes to the development of second tumors, there are few published data on the long-term risks of systemic chemotherapy in this patient population. Local delivery of chemotherapy could provide tumor control while minimizing the systemic risks.

Although intraocular retinoblastoma is increasingly treated with systemic carboplatin and other chemotherapeutic agents, little has been published on
the efficacy of carboplatin in controlling intraocular retinoblastoma, the plausibility of delivering carbo- 

pilot the first two issues using a transgenic mouse and the retinal toxicity of carboplatin. We have ad-

scribed a transgenic mouse model of hereditary retinoblastoma that has been characterized thoroughly and is similar to human reti-

oblastoma in anatomic, genetic, light, and electron microscopic, immunohistochemical, and ultrastruc-

tural features.26–30 Retinal toxicity of carboplatin was studied in rabbit eyes.

METHODS

All experiments in this study were conducted in accor-
dance with the ARVO Statement for the Use of Ani-
mals in Ophthalmic and Vision Research and the insti-
tutional guidelines regarding animal experimenta-
tion.

Intravitreal Carboplatin Injections in Transgenic Mice

Bilateral hereditary retinoblastoma develops in the mouse line used in this study because of the integra-
tion of a recombinant transgene comprised of simian virus 40 large T-antigen driven by the promoter for the beta-subunit of luteinizing hormone.26 Offspring bearing the transgene were identified by polymerase chain reaction analysis of tail DNA. Transgene-bearing mice, in which ocular tumors develop in more than 90% of eyes,26 were used for all subsequent experiments. Animals were anesthetized with intramuscular injections of ketamine and xylazine before intravitreal injections. Forty-eight 4-week-old, transgene-bearing mice were treated in the right eye with five intravitreal injections at biweekly intervals of either 0.01, 0.04, 0.1, 0.4, 1.0, 1.5, 4.0, or 10 μg of carboplatin in 2 μl of bal-

anced salt solution. The intravitreal injections were adminis-
tered using a 33-gauge needle inserted 1 mm posterior to the limbus and directed posterior to the lens under direct visualization through the pupil. A microvolume delivery pump was used to insure accu-
rate and reproducible delivery of the 2 μl volume. The left eyes were used as untreated controls. Six litter-
matched animals that served as controls were adminis-
tered 2 μl injections of balanced salt solution. After this, all animals underwent serial ophthalmologic ex-
naminations.

Histopathologic Study of Transgenic Mice

At 12 weeks after the last intravitreal carboplatin treat-
ment, all animals were killed with an overdose of keta-
mine and xylazine. Both eyes were enucleated, im-
mersed in 10% formalin, serially sectioned, and stained with hematoxylin and eosin. Light microscopic examination was performed in a masked fashion on all histopathologic sections, noting the location and size of all tumors, as well as corneal, lenticular, and scleral changes. Software using Logit statistical model-
ing was used to calculate the dose-response curves.

Intravitreal Carboplatin Injections in Rabbits

Twelve rabbits ranging in weight from 2.2 to 3.8 kg were used for the study. Each rabbit was anesthetized with intramuscular injections of ketamine (25 mg/kg) and xylazine (25 mg/kg) before intravitreal injections. The pupils were dilated with topical 1% atropine and 2.5% neosynephrine. A 30-gauge needle was inserted into the vitreous cavity through the pars plana, and 0.1 ml of vitreous fluid was removed. Next, a 0.1 ml solution containing either 1, 10, 100, or 1000 μg of carboplatin in balanced salt solution was injected slowly into the mid-vitreous cavity under direct visual-
ization through the pupil, with care taken not to touch the crystalline lens.

Electrophysiology in Rabbit Eyes

Rabbits were examined by electroretinogram (ERG) before injection, and at 1, 7, 21, and up to 123 days after intravitreal injection of carboplatin. Each rabbit initially was anesthetized with intramuscular injections of ketamine (25 mg/kg) and xylazine (25 mg/kg), followed by continuous intravenous infusion of urethane (25 mg/ml per hour) and flaxedil (10 mg/kg per hour) in lactated Ringer’s solution through the femoral vein. Animals were artificially respired. Pupils were dilated with topical 1% atropine and 2.5% neo-
synephrine. A contact lens with an 8 mm pupil was used to protect the cornea. The ERG was performed by inserting a 30-gauge hypodermic needle into the anterior chamber. The indifferent pole was placed subcutaneously near the recorded eye, and the animal was grounded by an electrode on the head. The ERG was amplified and displayed on an oscilloscope; the low-frequency cutoff was set at 10 Hz, and the high-

<table>
<thead>
<tr>
<th>Carboplatin (μg)</th>
<th>Number of Eyes</th>
<th>Number of Eyes With Complete Tumor Control (%)</th>
</tr>
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<tbody>
<tr>
<td>0.01</td>
<td>8</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0.04</td>
<td>3</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0.1</td>
<td>8</td>
<td>0 (0)</td>
</tr>
<tr>
<td>0.4</td>
<td>3</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1.0</td>
<td>5</td>
<td>0 (0)</td>
</tr>
<tr>
<td>1.5</td>
<td>6</td>
<td>3 (50)</td>
</tr>
<tr>
<td>4.0</td>
<td>9</td>
<td>9 (100)</td>
</tr>
<tr>
<td>10.0</td>
<td>6</td>
<td>6 (100)</td>
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frequency cutoff was set at 10 kHz. A Texas Instruments (Houston, TX) 960A minicomputer was used for off-line signal averaging. The stimulus was obtained from a 150-watt Xenon lamp bulb. Luminance of the full-intensity stimulus was $8.6 \times 10^6$ cd/m$^2$, and neutral-density filters were used to attenuate the intensity. The stimulus was presented in Maxwell view with the last lens subtending 26.5° at the cornea. Stimulus duration was 300 microseconds, and the interstimulus interval was 3.2 seconds.

**Histopathologic Study of Rabbit Eyes**

At various intervals after carboplatin injection, the rabbits were killed with overdoses of ketamine and xylazine. Treated eyes were enucleated, immersed in 10% formalin, serially sectioned, stained with hematoxylin and eosin, and examined by light microscopy. A portion of selected specimens was fixed in glutaraldehyde and embedded in plastic for electron microscopy.

**RESULTS**

Intravitreal injections of carboplatin inhibited tumor development in a dose-dependent manner in...
TABLE 2. Changes in the Electroretinogram Induced by Intravitreal Injection of Carboplatin in Rabbit Eyes

<table>
<thead>
<tr>
<th>Day</th>
<th>1 µg</th>
<th>10 µg</th>
<th>100 µg</th>
<th>1000 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preinjection</td>
<td>Preinjection</td>
<td>Preinjection</td>
<td>Preinjection</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>Normal</td>
<td>Normal</td>
<td>Negative b-wave</td>
<td>Negative b-wave</td>
</tr>
<tr>
<td>7</td>
<td>Normal</td>
<td>Normal</td>
<td>Negative b-wave</td>
<td>Flat</td>
</tr>
<tr>
<td>21</td>
<td>Normal</td>
<td>Negative b-wave</td>
<td>Flat</td>
<td>Flat</td>
</tr>
<tr>
<td>123</td>
<td>Normal</td>
<td>Not done</td>
<td>Not done</td>
<td>Not done</td>
</tr>
</tbody>
</table>

Intraocular Carboplatin Therapy in Transgenic Retinoblastoma transgenic retinoblastoma mice (Table 1). Histopathologic examination at 12 weeks after treatment revealed that all untreated control eyes and placebo control eyes administered injections of balanced salt solution had massive intraocular tumors (Fig. 1). Three (50%) of six eyes receiving 1.5 µg doses, and all eyes receiving 4 µg or higher doses of carboplatin, had complete absence of tumor on histopathologic examination at 12 weeks after treatment (Fig. 1). The calculated dose of carboplatin that resulted in complete tumor control in 50% of eyes (TCD50) was 1.4 µg (Fig. 2). There was no histopathologic evidence of extraocular extension of tumor through the injection sites in any of the treated eyes.

Electrophysiologic and histopathologic evidence of retinal toxicity for carboplatin was found to be dose-dependent in rabbits. Abnormalities on the ERG were observed after an intravitreal injection of 10 µg or more of carboplatin (Table 2, Figs. 3, 4). A 1000 µg dose of carboplatin resulted in a negative b-wave (P-III response) at 1 day after injection and extinction of the ERG response at 7 days after injection. A 10 µg injection of carboplatin resulted in a negative b-wave at 7 days after injection. This abnormality did not improve at 21 days after injection but did not progress to a flat ERG. These electrophysiologic abnormalities correlated with widespread disruption of the outer retina by light microscopy (Fig. 5) and altered synaptic profiles in the outer plexiform layer by electron microscopy (Fig. 5). A 1 µg injection of carboplatin caused no detectable electrophysiologic, histopathologic, or electron microscopic alterations up to 123 days after injection.

![Graph](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933194/ on 11/23/2018)

**FIGURE 4.** Stimulus intensity-response curves for 13 normal and 6 eyes that received 1 µg of carboplatin. Means and standard error of the means are plotted.

**FIGURE 5.** Histopathologic changes induced in the retina by carboplatin. (A) Intravitreal injection of 0.1 ml of physiological saline. Eye enucleated on day 21 after injection. Original magnification × 100. (B) Intravitreal injection of 10 µg of carboplatin. Eye enucleated on day 21 when negative electroretinograms were recorded. Original magnification, ×160. (C) Electron micrograph of retina taken 3 hours after 1000 µg of intravitreal carboplatin. Note the changes in the outer segments. Calibration = 0.5 µm. RPE = retinal pigment epithelium; Ph = photoreceptors; OS = outer segment; ONL = outer nuclear layer; INL = inner nuclear layer; GCL = ganglion cell layer.
DISCUSSION

In this study, we used a transgenic murine model of hereditary retinoblastoma to demonstrate that carboplatin inhibits intraocular tumor growth in vivo in a dose-dependent manner and that serial intravitreal injections of carboplatin are effective in this animal system. We also characterized the retinal toxicity and established toxicity limits of intravitreal carboplatin injection in rabbit eyes.

There is much interest in carboplatin as a primary agent in the treatment of intraocular retinoblastoma.\textsuperscript{10–12} Preliminary studies suggest that platinum compounds may inhibit the growth of retinoblastoma in vivo.\textsuperscript{34} Our study supports the efficacy of carboplatin in vivo and demonstrates dose-dependent tumor inhibition. Carboplatin injections were initiated in animals at 4 weeks of age, before massive intraocular tumors were present. Further work is needed to confirm whether carboplatin also is effective in the presence of large, established, intraocular tumors. The mechanism of the antineoplastic effect involves covalent binding of the diamino platinum radical to DNA, which inhibits DNA replication and cell division.\textsuperscript{20} This also may represent the mechanism of action leading to direct retinal toxicity. In addition, 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{92}

Because of the potential risks of systemic chemotherapy\textsuperscript{17,19–22} and the success of local drug delivery in other ocular diseases,\textsuperscript{38} we investigated the effectiveness of local chemotherapy using serial intravitreal injections of carboplatin. This therapeutic approach was effective in our animal model and suggested that local chemotherapy might provide an alternative to systemic chemotherapy, thereby minimizing the potential systemic toxicity. Intravitreal injection was chosen in this study to deliver a controlled amount of drug into the vitreous cavity. However, other methods of local drug delivery, such as topical drops,\textsuperscript{34} collagen corneal shields,\textsuperscript{35,36} and subconjunctival injection,\textsuperscript{37} might be considered in this disease. Future studies are needed to determine the usefulness of these systems for intraocular delivery of carboplatin.

Carboplatin was found to cause retinal toxicity in rabbit eyes in a dose-dependent manner. Toxicity was seen at carboplatin doses of 10 μg or more and consisted of cellular damage and synaptic disruption in the outer retinal layers. These alterations correlated with abnormalities of the electroretinogram. One-microgram injections were nontoxic. This finding is in agreement with results of previous studies that cisplatin, which is approximately 10-fold more cytotoxic than carboplatin,\textsuperscript{90} is nontoxic in rabbit eyes at doses up to 0.1 μg.\textsuperscript{38} A direct extrapolation of retinal toxicity from the rabbit eye model to the murine tumor model (or, for that matter, to the human eye) is not possible. Because of intraocular volumes and vascular supply, final toxicity studies most likely will require species-specific evaluation.

A major problem in studying new therapies for retinoblastoma has been the lack of an acceptable animal model. Previous animal models have included nude mouse xenografts\textsuperscript{39,40} and human adenovirus type 12-induced rodent tumors.\textsuperscript{41,42} However, both models have substantial anatomic, histopathologic, immunologic, and genetic differences from human retinoblastoma. One advantage of the current study is the use of a transgenic mouse model of hereditary retinoblastoma that has been characterized thoroughly and is remarkably similar to human retinoblastoma in terms of anatomic, genetic, light and electron microscopic, immunohistochemical, and ultrastructural features.\textsuperscript{20–30} These similarities, along with the autosomal dominant transmission and high penetrance rate of spontaneous bilateral intraocular tumors, makes this an excellent animal model for testing potential treatment modalities.\textsuperscript{43}

Chemotherapy for intraocular retinoblastoma may offer several improvements over conventional therapy, including the reduction of tumor bulk and the avoidance of radiation risk, as well as the salvaging of some eyes with large tumors that otherwise would require enucleation.\textsuperscript{4,5,10–12} Carboplatin is used widely in pediatric oncology\textsuperscript{44} and is an important component of most current regimens for intraocular retinoblastoma.\textsuperscript{10–12} We have confirmed that locally delivered carboplatin confers dose-dependent tumor inhibition in a transgenic murine model of retinoblastoma. Because of difficulties associated with intravitreal drug delivery within this murine model, the absolute TCD\textsubscript{50} may be lower than the 1.4 μg determined in this study. The efficacy of local carboplatin delivery in this model suggests that local chemotherapy for intraocular retinoblastoma may provide tumor control while minimizing the systemic side effects and mutagenic potential. Pharmacokinetic studies are necessary to evaluate various potential systems for intraocular carboplatin delivery.

**Key Words**

carboplatin, chemotherapy, retinoblastoma, toxicity studies, transgenic animal models

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