In Vitro Efficacy of Carboplatin and Hyperthermia in a Murine Retinoblastoma Cell Line

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Purpose. To determine the cell-killing activity of varying doses of carboplatin, graded hyperthermia, and the combination of carboplatin and hyperthermia in the treatment of a transgenic murine retinoblastoma cell line.

Methods. Replicate cell wells (more than six wells per dose point) from an established transgenic murine retinoblastoma cell line (Rb-6) were exposed to a single application of hyperthermia for 15, 30, 60, and 120 minutes at temperatures of 37°C (control), 40°C, and 43°C. Carboplatin dose response treatment was studied at doses of 2000, 1000, 500, 400, 300, 200, 100, and 50 ng per well. Combined treatment studies used these carboplatin dosages with each of the graded hyperthermia exposure temperatures at each exposure time. At 24 hours, all wells were pulsed with 3H-thymidine for 24 hours, washed three times, harvested, and counted. Raw counts (3H-thymidine) were fitted to a linear regression model to calculate the lethal dose for 50% (LD50) of cells.

Results. The LD50 for carboplatin exposure at 37°C occurred at 542 ng. The LD50 for hyperthermia at 40°C occurred at 90 minutes and at 43°C it occurred at 62 minutes. Combined hyperthermia and carboplatin exposure yielded a synergistic interaction with an LD50 of 327 ng at 43°C for 30 minutes. Determination of a thermal enhancement ratio yielded an enhancement range of 1.1 to 25.8.

Conclusions. The synergistic cytocidal interaction of heat and carboplatin in a transgenic murine retinoblastoma cell line has been established in this study. The increased thermal enhancement ratio documents the potential utility of combined treatment applications in reducing treatment levels of single-modality therapy, potentially allowing for a decrease in treatment-related morbidity.

Retinoblastoma is the most common primary intraocular malignancy of childhood.1 Patients with the heritable form of the disease typically develop bilateral, multiple tumor foci at an early age, whereas nonheritable forms are uniformly solitary and unilateral.2

In the absence of treatment, tumors are fatal in virtually all patients. Conventional therapy in unilateral retinoblastoma has been enucleation of the affected eye. Historically, in bilateral cases, attempts at globe-conserving therapy have relied on external beam radiotherapy for larger tumors and on photocoagulation, cryotherapy, or plaque radiotherapy for smaller tumors amenable to these focal treatments.3,4 Survival rates higher than 90% are reported with traditional treatment modalities.5–7 A recent trend toward earlier diagnosis of retinoblastoma and continued improvements in conservative treatments have led to a decrease in the frequency of enucleation for bilateral and unilateral retinoblastoma.8–10 Current single-modality therapy, especially that using external beam radiotherapy, is associated with considerable morbidity in the form of second tumors,11–14 recurrence,15 and complications of ionizing radiation.
In Vitro Carboplatin and Hyperthermia Therapy in Murine Retinoblastoma

Chemotherapy plays a major role in the management of extracellular retinoblastoma. Although cyclophosphamide and vincristine are the most commonly used chemotherapeutic agents in this disease, platinum-containing compounds are increasingly employed because of decreased associated toxicity. Toxicity studies of treatment with carboplatin have shown less associated nephro- and ototoxicity than treatment with cisplatinum. The contribution of chemotherapy monolytically used chemotherapeutic agents in this disease, clophosphamide and vincristine are the most commonly employed because of decreased associated toxicity. Toxicity studies of treatment with carboplatin have shown less associated nephro- and ototoxicity than treatment with cisplatinum. The contribution of chemotherapy in treatment of local, micrometastatic, and recurrent retinoblastoma has been difficult to evaluate because of ethical, biologic, and statistical obstacles to this clinical research.

Alternatively, multimodality therapy has the potential of minimizing treatment-related morbidity. The synergistic effect of platinum-containing chemotherapeutic agents and hyperthermia has been recently established for some solid tumors. Results of a phase I clinical trial in pediatric solid tumors have shown that this treatment combination is well tolerated at conventional doses of carboplatin. Further, recent studies have documented, in animal models and in human clinical trials, the efficacy of chemoreductive therapy in the management of retinoblastoma. We have used established cell lines from a recently developed transgenic mouse model of heritable retinoblastoma to evaluate the efficacy of focally delivered carboplatin, hyperthermia, and combined treatment in the cure of murine retinoblastoma.

METHODS

Retinoblastoma Cell Line

Retinoblastoma cells were obtained from transgenic mice with retinoblastoma that were originally developed by Dr. Jolene Windle (San Antonio Cancer Center, TX) and characterized by Drs. Joan O’Brien and Daniel Albert (Department of Ophthalmology, University of Wisconsin). These mice were obtained using a transgene containing the simian virus 40 (SV-40), early region, which includes large and small T antigens, and the luteinizing hormone beta subunit promoter. Transgenic mice were bred by mating founder mice with CB6F1/J mice. Bilateral retinoblastoma developed in all offspring possessing the transgene.

The Rb-6 tumor cell line was obtained from the tumor-containing mouse eyes, using a modification of the techniques described by Kivela et al. Tumor cells were obtained by enzymatic digestion of Rb tumor tissue recovered from 4-month-old mice, using a technique previously described for ocular tumor cells by Ksander et al., and were cultured in 96-well culture plates for approximately 1 week. Proliferating tumor cells were gradually expanded and cultured in standard tissue culture flasks.

The characteristics of tumor cells derived from Rb transgenic mice have been described previously by Kivela et al. Briefly, these tumor cells express synaptophysin and individual neurofilament proteins, indicating their neuronal nature. The antigenic profile of Rb-6 cells suggests a close relationship with neurons of the inner nuclear layer. The Rb-6 tumor cells have morphology similar to human Rb cell lines Y-79 and WERI.

Treatment and Survival of Rb-6 Cells

Rb-6 cells treated with carboplatin, hyperthermia, or both were assessed for survival by an assay that measures uptake of tritiated thymidine in vitro. All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rb-6 cells (0.03 X 10^6 cells/ml) were cultured in 96-well culture plates in a total volume of 0.2 ml RPMI 1640 media (BioWhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, 1% HEPES, 1% penicillin--streptomycin, 1% glutamine, and 0.1% 2-ME. Tumor cells were grown to subconfluent cultures in a humidified 5% CO2--95% air atmosphere at 37°C and treated, as described later, for 72 hours. For the last 24 hours of treatment, 0.025 µl 3H-thymidine (specific activity 20 µCi/ml; NEN Dupont, Wilmington, DE) was added to culture wells. Tumor cells were harvested with a PHD automated cell harvester (Cambridge Technology, Cambridge, MA) and incorporated of 3H-thymidine was measured using a Beckman LS 5801 (Fullerton, CA) automated scintillation counter. As a positive control in these experiments, incorporation of 3H-thymidine was determined in Rb-6 tumor cells that received neither carboplatin nor hyperthermia treatment.

Studies using a clonal dilution assay and trypan blue vital staining for cell survival were also performed but yielded less reproducible data because of cell clumping and are not included in this report.

Carboplatin (Bristol–Myers–Squibb Pharmaceutical Research Institute, Hillside, NJ), in powder form, was dissolved in sterile balanced salt solution and diluted to produce the desired concentrations (50, 100, 200, 300, 400, 500, 1000, and 2000 ng/well or 250, 500, 1000, 1500, 2000, 2500, 5000 and 10,000 ng/ml). Hyperthermia treatment was applied by incubating cultures in a temperature-equilibrated humidified incubator at 40°C or 43°C for 15, 30, 60, or 120 minutes.

Cell-killing activities of increasing concentrations of carboplatin, increasing temperature, and increasing time of heating were assessed to arrive at a model for predicting cell counts from treatment combinations of carboplatin and hyperthermia. Replicates of greater than, or equal to, six wells per treatment point were obtained for each experiment. All experiments were repeated at least three times. All raw 3H-thymidine counts per well were directly integrated into the
FIGURE 1. Dose-response curves for Rb-6 cells exposed to varying concentrations of carboplatin and to 40°C hyperthermia at graded exposure times (see scale for heating times). Data are reported as median cell counts, using ³H-thymidine incorporation and automated cell harvesting (minimum six replicates per well).

FIGURE 2. Dose-response curves for Rb-6 cells exposed to varying concentrations of carboplatin and to 43°C hyperthermia at graded exposure times (see scale for heating times). Data are reported as median cell counts, using ³H-thymidine incorporation and automated cell harvesting (minimum six replicates per well).

model, as described below. Using regression analysis, the LD₅₀ was calculated for single-modality therapy for carboplatin alone and hyperthermia at 40°C and 43°C for each time point. Combined modality therapy was evaluated using the carboplatin dose response points combined with hyperthermia at 40°C and 43°C at each time point.

Statistical Analysis
We used regression analysis to model the effects of carboplatin concentration and increased heat (temperature and heating time) on the reproductive integrity of retinoblastoma cells. Models fitted with the raw ³H-thymidine cell counts as the dependent variable produced normally distributed residuals but demonstrated severe heterogeneity of variance; whereas models fitted to square-root or log-transformed counts showed less heterogeneity of variance but produced residuals that were not normally distributed. Therefore, weighted least-squares regression analysis methods were used to estimate the effects of carboplatin concentration (C), temperature of heating (H), and length of heating (T). Linear and quadratic carboplatin terms were included. Interactions between these factors were examined. Because the effect of increased temperature, 43°C over 40°C, is not significant when the time of heating is 0, temperature was included as a variable only in the model in interaction with time of heating (that is, H·T and C·H·T were allowed into the model; terms H, C·H were excluded). Median lethal doses were calculated for each variable, alone and in combination, to yield enhancement ratios with model coefficients. The thermal enhancement ratio (TER) was calculated for each hyperthermic time point and represents the degree of interaction of carboplatin exposure and of hyperthermia. A TER higher than 1 is associated with a synergistic interaction of the combined treatment modalities.

RESULTS
Rb-6 retinoblastoma cell lines are responsive in a time-dependent fashion to treatment with carboplatin, hyperthermia, or both at 40°C and at 43°C. Carboplatin is cytotoxic to Rb-6 cells in a dose-dependent manner (Figs. 1, 2; Tables 1, 2), with an LD₅₀ of 542 ng. Enhanced cell death, measured by decreased ³H-thymidine incorporation, is seen with increasing temperature and with increasing duration of exposure to hyperthermia, with an LD₅₀ of 90 minutes at 40°C and of 62 minutes at 43°C (Figs. 1, 2, Table 1). The regression model on which these LD₅₀s are based is presented in Table 3. Incubation at 43°C for 120 minutes resulted in complete tumor kill at all doses of carboplatin (Fig.

<table>
<thead>
<tr>
<th>Treatment Modality</th>
<th>LD₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboplatin alone</td>
<td>542 ng</td>
</tr>
<tr>
<td>40°C hyperthermia alone</td>
<td>90 minutes</td>
</tr>
<tr>
<td>43°C hyperthermia alone</td>
<td>62 minutes</td>
</tr>
</tbody>
</table>

LD₅₀ = lethal dose for 50% of cells.

TABLE 1. Single Modality Treatment With Carboplatin or Hyperthermia
TABLE 2. Combined Modality Treatment With Carboplatin and Hyperthermia

<table>
<thead>
<tr>
<th>Heat (°C)</th>
<th>Exposure Time (minutes)</th>
<th>Carboplatin LD$_{50}$ (ng)</th>
<th>Thermal Enhancement Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>40°C</td>
<td>15</td>
<td>494</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>437</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>280</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>*</td>
<td>NA</td>
</tr>
<tr>
<td>43°C</td>
<td>15</td>
<td>443</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>327</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>21</td>
<td>25.8</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>*</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA = not applicable; LD$_{50}$ = lethal dose for 50% of cells.
* The model predicts >50% cell death with no carboplatin at 120 minutes.

2). All three factors studied had highly significant effects on cell counts (all $P$ values < 0.001). Combined modality treatment with hyperthermia and carboplatin demonstrated increased tumoricidal synergistic activity as duration of exposure to hyperthermia increased. The TER increased to a maximum of 25.8 when cells were exposed to 43°C for 60 minutes (Table 2). For example, combined therapy yielded an LD$_{50}$ of 327 ng at 43°C for 30 minutes.

DISCUSSION

Recent trends toward more conservative, sight-preserving management of retinoblastoma are the result of earlier diagnosis, improved treatment techniques, and findings in studies that have established the relative indications and limitations of alternatives to enucleation.28–31,40 However, the role of chemotherapy remains to be established definitively, and the indications for treatment have not been clearly delineated.

Multimodality treatment, combining hyperthermia with chemotherapy, has the potential to increase efficacy while minimizing treatment-associated morbidity, especially when both treatment modalities are focally delivered.22–30,36 Hyperthermia induced by several different heating mechanisms has been efficacious in the treatment of ocular tumors.28–30,46–50 Effects of hyperthermia include damage to the microtubular system,51 increased vascular permeability,52 increased intracellular space, vacuole formation, clumping of chromatin, breaks in the cell membrane, and swelling and collapse of cells.49 Increased temperature or duration of heat exposure results in increased tumoricidal activity against transgenic murine retinoblastoma cell lines, perhaps because of increased DNA cross-linkage, increased permeability, or decreased DNA repair.53

The TER is used to describe the enhanced cellular killing that results when hyperthermia is combined with other treatment modalities. A synergistic interaction between treatment modalities is found when the TER is significantly higher than 1. Until recently, the interaction of various treatment modalities in the management of retinoblastoma was difficult to assess because of the lack of an appropriate experimental animal model. The development of a transgenic murine model of heritable retinoblastoma that is genetically, histopathologically, ultrastructurally, and behaviorally similar to human retinoblastoma has allowed for evaluation of drug efficacy and interactions.32–38 We have used established cell lines isolated from bilateral murine retinoblastoma from a transgenic SV40 T-antigen-positive mouse as a model to evaluate single and multiple modality therapies for retinoblastoma in vitro.

Cisplatinum has shown particular promise for hy-

**TABLE 3. Weighted Least-Squares Analysis of Cell Counts**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient ± Standard Error</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear carboplatin concentration (ng)</td>
<td>$-157 ± 3.3$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quadratic carboplatin term</td>
<td>$0.0468 ± 0.001$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time of heating (minutes)</td>
<td>$4126.1 ± 247.8$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time·heat temperature (min°C)*</td>
<td>$-123.0 ± 5.7$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Linear carboplatin concentration·time (ng-min)</td>
<td>$1.076 ± 0.045$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quadratic carboplatin concentration·time</td>
<td>$-0.00154 ± 0.00008$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Linear carboplatin concentration·heating time (ng/min°C)</td>
<td>†</td>
<td></td>
</tr>
<tr>
<td>Quadratic carboplatin concentration·heating temperature·heating time</td>
<td>$0.0000295 ± 0.000002$</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Constant</td>
<td>143,535.1</td>
<td></td>
</tr>
</tbody>
</table>

* The statistical significance of this interaction term in the model indicates that the effect on cell counts of a specific time of heating is different for cells heated to 43°C from those heated to 40°C.
† Excluded from model due to multicollinearity.

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thermic chemosensitization because the tumoricidal effect is enhanced at all elevated temperatures, with synergism reached at higher than, or equal to, 42°C. Significant renal injury can result from systemic cisplatinumum therapy and hyperthermia, even if hyperthermia is delivered locally. Local therapy with both modalities in the treatment of melanoma-bearing mice significantly reduced renal toxicity while reducing tumor mass and improving prognosis.

Carboplatin, an analogue of cisplatinumum, has the same mechanism of action (creation of inter- and intrastrand linkages that impair cell replication), superior penetration into brain tissue, and decreased associated nephro- and ototoxicity. A synergistic thermal enhancement of carboplatin tumoricidal activity has been shown to be superior to that found with cisplatinumum, with well-tolerated toxicity in a phase I clinical trial of whole-body hyperthermia and in a study of retinotoxicity after intravitreal injection. Rb-6 transgenic murine retinoblastoma cell lines are sensitive in a dose-dependent manner to treatment with focally delivered carboplatin in vitro.

We demonstrate a TER of 1.1 to 25.8 when murine retinoblastoma cells are treated in vitro with hyperthermia and carboplatin—a result comparable to the TER of ~3.5 obtained with combined treatment of human leukemia cells. The synergistic interaction of carboplatin and hyperthermia increased with increasing heat exposure. The mechanism of synergistic tumoricidal activity may involve an increase in membrane permeability with resulting increased drug uptake and increased reaction rate.

These data indicate that the relatively less toxic chemotherapeutic agent carboplatin exhibits synergistic cell killing when coadministered with hyperthermia to cell cultures of murine retinoblastoma. The LD_{50} of the carboplatin-treated eyes was 542 ng, a dose comparable to the tumor-control dose’s reported LD_{50} of 1400 ng, in murine transgenic retinoblastoma tumors treated with intravitreal injection of carboplatin. Retinal toxicity for intravitreal injection is not seen at carboplatin doses less than 10 μg. As might be expected, the LD_{50} occurs at a lower dose in cell lines with direct in vitro exposure than it does in treatments of the murine ocular retinoblastoma tumors in vivo.

The combination of carboplatin and hyperthermia, if locally administered, may increase the efficacy of conservative treatment of human intraocular retinoblastoma while decreasing systemic toxicity. Several mechanisms of local delivery of heat and chemotherapeutics are being investigated. Further studies of toxicity and efficacy in vivo, using the transgenic mouse model of retinoblastoma, would provide additional information regarding the application of combined chemotherapy and hyperthermia in the treatment of retinoblastoma.

Key Words
cell modeling, chemotherapy, hyperthermia, retinoblastoma, transgenic animals

Acknowledgments
The authors thank Dr. Jolene Windle for development of the transgenic mouse; Dr. Daniel Albert for characterization of the pathology and modeling for use of the transgenic mouse; and Dr. Steve Schoenike for assistance in pediatric pharmacology, particularly regarding the pharmacokinetics of carboplatin; and Bristol-Myers-Squibb Pharmaceutical Research Institute, Hillside, New Jersey, for graciously providing carboplatin.

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