Subconjunctival Nanoparticle Carboplatin in the Treatment of Murine Retinoblastoma

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Objective: To evaluate the efficacy of subconjunctival nanoparticle carboplatin in the treatment of transgenic murine retinoblastoma.

Methods: Dendrimeric nanoparticles loaded with carboplatin were prepared. Forty LHβ-Tag mice were randomly assigned into 4 groups and treated at 10 weeks of age. Each mouse received a single subconjunctival injection in one eye, and the opposite eye was left untreated as a control. Group 1 (high-dose nanoparticle carboplatin) received 37.5 mg/mL of nanoparticle carboplatin; group 2 (low-dose nanoparticle carboplatin) received 10 mg/mL of nanoparticle carboplatin; group 3 (conventional carboplatin) received 10 mg/mL of carboplatin in aqueous solution; and group 4 (phosphate-buffered saline) received phosphate-buffered saline. Mice were killed on day 22 after treatment. Eyes were serially sectioned, and retinal tumor burden was quantified by histopathologic analysis.

Results: Mean tumor burden in the treated eyes was significantly smaller compared with the untreated eyes in the same mice in both nanoparticle carboplatin groups (group 1, \( P = .02 \); group 2, \( P = .02 \)) and the treated eyes in the conventional carboplatin group (group 1 vs group 3, \( P < .01 \); group 2 vs group 3, \( P = .01 \)) and phosphate-buffered saline group (group 1 vs group 4, \( P < .01 \); group 2 vs group 4, \( P = .01 \)). The untreated eyes in the high-dose nanoparticle carboplatin group showed significantly smaller tumor mass compared with the conventional carboplatin (\( P = .03 \)) and PBS (\( P = .04 \)) groups. No toxic effects were observed in any of the groups.

Conclusion: A single injection of subconjunctival nanoparticle carboplatin was effective in the treatment of transgenic murine retinoblastoma, with no associated toxic effects. The higher dose of subconjunctival nanoparticle carboplatin decreased the tumor burden in the contralateral eye.

Clinical Relevance: This model provides a basis to test carboplatin nanoparticles for the treatment of human retinoblastoma.


Retinoblastoma is the most common eye cancer in children, accounting for 11% of all infant cancers and 3% of cancers developing in children younger than 15 years. There are 300 new cases of retinoblastoma per year in the United States.1 For unilateral, sporadic cases, enucleation is the treatment of choice, whereas other treatment modalities are considered for the familial, bilateral cases.2,3 Radiation therapy and/or systemic chemotherapy with carboplatin, etoposide phosphate, and vincristine sulfate are preferred treatment options, which are associated with increased incidence of systemic adverse effects and secondary malignancies.4-8

To minimize the systemic complications, local delivery of the chemotherapeutic agent to the eye has been proposed.9,10 Subconjunctival injection of carboplatin initially showed promising results,11 followed by reports of local toxic effects of the periocular carboplatin, such as orbital fat necrosis, ocular motility changes,12 and ischemic necrosis and atrophy of the optic nerve.13 It was assumed that these toxic effects were caused by the rapid dispersion of the aqueous solution of carboplatin to the surrounding orbital tissue with subsequent undesired adverse effects. Subconjunctival injection of carboplatin in fibrin sealant showed the advantage of sustained release with longer duration and minimal local toxic effects.14

Nanoparticles are widely used in the medical field as a therapeutic or diagnostic agent, have lower therapeutic toxic-
Dendrimers are a novel class of hyperbranched polymers that are nanosized with a number of surface functional groups. Poly(amideamine) (PAMAM) dendrimers have been widely used as delivery systems for various therapeutics in cancer and arthritis. In this study, we evaluated the therapeutic effects of a single subconjunctival injection of carboplatin-loaded PAMAM dendrimeric nanoparticles in murine transgenic retinoblastoma.

**METHODS**

**ANIMALS**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Emory University and conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The LHβ-Tag mouse model used in this study carries a transgene composed of the coding region of the simian virus 40 large T antigen (Tag) driven by the human luteinizing hormone β-subunit promoter gene (LHβ), developing multifocal, bilateral retinal tumors analogous to human retinoblastoma. Mice in this study were treated at 10 weeks of age.

**PREPARATION AND CHARACTERIZATION OF CARBOPLATIN-LOADED DENDRIMERIC NANOPARTICLES**

For preparing carboplatin-loaded nanoparticles, half-generation PAMAM dendrimer (G3.5 PAMAM, 32 carboxyl end groups) was used. First, a weighed quantity of carboplatin (27.94 mg) was dissolved in 20 mL of deionized water and stirred well until a clear solution was obtained. Then 0.375 mL of G3.5 PAMAM solution (30.43 mg) was dissolved in 4 mL of deionized water and exposed to a stream of nitrogen for 30 minutes to evaporate the trace amount of methanol. The dendrimer solution was slowly added to the drug solution and stirred for 24 hours at room temperature. The stirred solution was dialyzed against deionized water for 4 hours to remove the unentrapped drug. The final dialysate was lyophilized and characterized for particle size and drug loading.

**TREATMENTS**

Mice were randomly assigned into 4 groups of 10 animals each. Mice were first anesthetized with a mixture of ketamine hydrochloride and xylazine hydrochloride and then received a single 30-μL injection of each solution in the superior temporal subconjunctival space of the right eye, and the left eye was untreated as an internal control. Group 1 (high-dose nanoparticle carboplatin group) received 10 mg/mL of nanoparticle carboplatin, the same concentration as the conventional carboplatin solution. Conventional carboplatin solution (10 mg/mL) was prepared by adding carboplatin (C2528; Sigma-Aldrich, St Louis, Missouri) to phosphate-buffered saline (PBS). Group 3 (conventional group) received 10 mg/mL of carboplatin in aqueous solution, and group 4 (PBS group) received 30 μL of PBS. Mice were killed on day 22, and both eyes were enucleated.

**DETERMINATION OF OCULAR TUMOR BURDEN**

After enucleation, the eyes were fixed in 10% neutral-buffered formalin, processed routinely, and serially sectioned and stained with hematoxylin-eosin. The slides were obtained from at least 10 different levels throughout each eye. For each eye, 1 section from each level was examined by light microscopy and all tumor foci were identified and digitally imaged at ×100 magnification. The cross-sectional area of each tumor focus was measured in square pixels with the ImageJ program, and the total area of all tumor foci was divided by the number of levels examined to calculate the mean tumor burden.

For statistical analysis, an unpaired, 2-tailed t test was used to compare mean ocular tumor burden per level among the 4 groups. Statistically significant difference was defined as P < .05.

**RESULTS**

Carboplatin-loaded dendrimeric nanoparticles had a mean (SD) particle size of 258 (5.7) nm with a very narrow polydispersity index of 0.072 (0.05). Carboplatin content was found to be 0.475 mg/mg of nanoparticles (47.54% weight for weight drug loading) as estimated by a previously described high-performance liquid chromatography method.

There were no clinically significant complications after subconjunctival injection, and there were no toxic effects in the retina, optic nerve, or other ocular tissues. The results of the tumor burden analysis are summarized in **Figure 2** and **Figure 3**. The mean tumor burden in treated eyes in group 1 and group 2 was significantly smaller than that of untreated eyes from the same mice (mean [SD], group 1, 82 064 [85 503] vs 288 634 [89 052] square pixels; P = .02; group 2, 198 392 [117 373] vs 992 633 [126 795] square pixels; P = .02). One of the eyes in group 1 demonstrated zero tumor burden. Eyes
treated with carboplatin in aqueous solution (group 3) or PBS (group 4) did not show significant difference compared with the untreated contralateral eyes (mean [SD], group 3, 724 956 [186 433] vs 864 264 [305 110] square pixels; \(P = .24\); group 4, 887 485 [165 861] vs 1 001 467 [117 121] square pixels; \(P = .53\)). The mean tumor burden in eyes treated with both high- and low-dose nanoparticle carboplatin was significantly smaller than eyes treated with carboplatin in aqueous solution (group 1 vs group 3, \(P < .01\); group 2 vs group 3, \(P = .01\)) and in the PBS group (group 1 vs group 4, \(P < .01\); group 2 vs group 4, \(P = .01\)). There was no significant difference in mean tumor burden between eyes treated with high-dose nanoparticle carboplatin and low-dose nanoparticle carboplatin (group 1 vs group 2, \(P = .07\)). A single subconjunctival injection of conventional carboplatin did not show a significant decrease in tumor burden compared with the PBS group (group 3 vs group 4, \(P = .53\)).

The untreated eyes in the high-dose nanoparticle carboplatin group showed statistically significant smaller tumor mass compared with the untreated eyes in the conventional carboplatin group and PBS group (group 1 vs group 3, \(P = .03\); group 1 vs group 4, \(P = .04\)).

**COMMENT**

In this study, we evaluated the therapeutic effect of a single subconjunctival injection of nanoparticle carboplatin in murine transgenic retinoblastoma. Our results showed that the subconjunctival nanoparticle carboplatin was more effective compared with the carboplatin in aqueous solution.

To enhance the delivery of the drug to the eye, we used the nanoparticle form of carboplatin. Advantages of nanoparticle-based drug delivery are that it improves the solubility of poorly water-soluble drugs, prolongs the half-life of drug systemic circulation by reducing immunogenicity, releases drugs at a sustained rate, lowers the frequency of administration, delivers drugs in a target manner to minimize systemic adverse effects, and enables delivery of 2 or more drugs simultaneously for combination therapy to generate a synergistic effect and suppress drug resistance.15,21 We used PAMAM dendrimer nanoparticles with a diameter of 258 nm. The dose of carboplatin in this study was based on the report by Van Quill and coworkers,14 who injected carboplatin in fibrin sealant into the subconjunctival space of the LH9252-Tag mice using 2 different doses (low dose, 37.5 mg/mL; high dose, 75 mg/mL). The low-dose group showed tumor regression in 91% of the eyes, whereas the high-dose group showed a similar treatment effect with severe toxic effects.14 Therefore, we chose the concentration of 37.5 mg/mL for our high-dose nanoparticle carboplatin group. The low-dose nanoparticle carboplatin group used the same concentration as the carboplatin in aqueous media at its highest stable concentration of 10 mg/mL.

The results showed that the eyes treated with both high- and low-dose nanoparticle carboplatin showed significant reduction in the mean tumor burden compared with the untreated contralateral eyes. Even with
the same concentration (10 mg/mL), nanoparticle carboplatin showed smaller tumor mass compared with the carboplatin in aqueous solution. This observation can be explained on the basis of prolonged retention of nanoparticles and sustained drug delivery when compared with plain drug.

In a previous study, we investigated the influence of particle size on disposition of nanoparticles following periorcular injection in a rodent model. For 20-nm nanoparticles, at 6 hours after periorcular injection, we observed particle accumulation in the spleen, liver, and cervical, axillary, and mesenteric lymph nodes of the animals, but not in the intraocular tissues, including the retina and vitreous. Further, there was no detectable transport of 20-nm nanoparticles across excised bovine sclera-choroid–retinal pigment epithelium in 24 hours. Thus, small nanoparticles of 20 nm are not transported well across the sclera to the retina and they are rapidly cleared by lymph and/or blood circulation in the periocular space. We observed that only 15% of the administered dose of 20-nm particles is retained in the periocular space after 24 hours in rodents. However, when 200-nm particles were assessed in the same study, nearly the entire dose of particles was retained at the site of administration for at least 2 months. Therefore, the dendrimer particles administered in this study (258 nm) are not likely to cross the sclera-choroid–retinal pigment epithelium in their original form. However, the drug released from these systems can be transported across ocular as well as systemic barriers.

Since the nanoparticles used in this study are aggregates of dendrimer molecules present at less than 5 nm, we cannot rule out the possibility that the administered dendrimer aggregates dissociate slowly into drug-dendrimer particles less than 20 nm, thereby entering the circulatory system as well as intraocular tissues. It is anticipated that PAMAM nanoparticle aggregates dissociate eventually, releasing individual nanoparticles. These small particles are expected to be cleared by blood and lymphatic systems. Also, there is a possibility that the smaller particles may traverse sclera to access the intraocular tissues. Ultimately, once in their molecular form, G3.5 PAMAM dendrimers are expected to be removed from the body via glomerular filtration.

We observed that the untreated left eyes in the high-dose nanoparticle carboplatin group showed significantly smaller mean tumor burden compared with the untreated eyes in the conventional carboplatin group and in the PBS group. Tsui and coworkers investigated the effect of subconjuunctival topotecan in fibrin sealant for the treatment of transgenic murine retinoblastoma. In mice treated with topotecan, tumor burden in treated eyes and in untreated contralateral eyes did not differ significantly. However, comparison of mean tumor burden in both eyes from topotecan-treated and from control mice demonstrated a statistically significant reduction in tumor burden (P = .04). Tsui and coworkers concluded that topotecan does not cross the sclera as efficiently as carboplatin in vivo because it is cleared by local vascularization at a significantly higher rate than carboplatin and the major route of drug delivery in this system is hematogenous rather than transcleral. Our results implicate a similar effect by the size of nanoparticle (>200 nm) and overloading of the nanoparticle carboplatin with higher dose. The higher concentration of nanoparticle carboplatin not only crossed directly through the sclera, but also was retained for a longer period, thus clearly by the local vascular supply to reach the contralateral untreated eye. This is further supported by an earlier study of ours wherein we observed that following administration of a high dose of celecoxib (3 mg/rat) in the periocular space, drug was detected in the plasma and the retina of the contralateral undosed eyes.

In summary, a single injection of subconjunctival nanoparticle carboplatin was effective in the treatment of transgenic murine retinoblastoma. A higher dose of subconjunctival nanoparticle carboplatin could reach and decrease the tumor in the untreated contralateral eye.

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REFERENCES

In Memoriam: Carl B. Camras, MD (1953-2009)

C arl B. Camras, MD, died peacefully at home on April 14, 2009, after a long battle with constrictive pericarditis. Dr Camras was a world-renowned glaucoma specialist and research scientist. His most widely recognized contribution to ophthalmology was the development of the prostaglandin analogues for the treatment of glaucoma.

Born in Glencoe, Illinois, on November 23, 1953, Carl began his work with prostaglandins as an undergraduate majoring in biochemistry at Yale University. He discovered that, in contrast to what was believed at the time, prostaglandins can lower intraocular pressure if given in low concentrations. In medical school at Columbia University, with convincing data and much perseverance, Camras persuaded one of his professors to take on the prostaglandin project. In collaboration with many others, the topical prostaglandin F_2alpha analogue latanoprost was developed. First approved in 1996, the prostaglandin analogues are now the most widely prescribed glaucoma medications in the world.

While pursuing the prostaglandin work, Dr Camras also completed his residency at the University of California--Los Angeles Jules Stein Eye Institute and a glaucoma fellowship at Mount Sinai School of Medicine in New York. He served on the faculty at Mount Sinai from 1983 to 1991, then he came to the University of Nebraska Medical Center in Omaha. Dr Camras became chairman of the Department of Ophthalmology and Visual Sciences in the year 2000 and led the department to new levels of excellence in research, teaching, and patient care. As a clinician and surgeon, Dr Camras attracted international referrals. As a teacher, he was so often recognized by his residents for his excellence that in 2008 they named the departmental teaching award in his honor.

But Carl Camras’ extraordinary professional accomplishments are only a small part of what made him so widely respected. His colleagues and coworkers consistently marveled at his kindness and generosity. Young students and indigent patients were treated with as much regard as any famous scientist or wealthy business leader. Carl was a maximalist who strove for excellence in every aspect of his life, and he did so with honesty, integrity, and the greatest respect for others. The greatest tribute that we can give Carl is for each of us to do the same.

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