Comparison of Long-Acting Bevacizumab Formulations in the Treatment of Choroidal Neovascularization in a Rat Model

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Abstract

Objective: The objective of this study was to compare the reduction in size of experimentally induced choroidal neovascularization (CNV) in rat eyes treated with *bevacizumab*, poly(ethylene-glycol) (PEG)-*bevacizumab* conjugate (b-PEG), and poly(lactic-co-glycolic acid) (PLGA)-encapsulated *bevacizumab* (b-PLGA).

Methods: Forty-eight eyes from 24 rats were divided into 4 groups of 12 eyes. In each group, 3 eyes were assigned to a treatment subgroup, each receiving a different injection—control, *bevacizumab*, b-PEG, and b-PLGA. In all eyes, laser photocoagulation was used to rupture Bruch's membrane. In group 1, laser was followed by injection, which was then followed by harvesting the rats to assess the CNV area. All 3 steps were separated by a 2-week interval. In groups 2, 3, and 4, injection preceded laser photocoagulation by a variable interval and all rats were harvested 2 weeks postlaser treatment. In group 2, laser and injection were separated by 2 weeks. In group 3, laser followed injection by 4 weeks. In group 4, laser followed injection by 6 weeks. The CNV area was measured for each subgroup and compared against its control. Pairwise comparisons were conducted to assess for statistically significant differences between subgroups.

Results: All subgroups in groups 1, 2, and 4 showed statistically significant reduction of CNV area ($P < 0.05$). In group 3, the b-PEG and b-PLGA subgroups showed a 9.0% ($P = 0.384$) and 20.3% ($P = 0.077$) reduction in CNV area versus control, whereas there was no reduction in CNV area in the *bevacizumab* subgroup. However, this was not found to be statistically significant. In group 4, b-PEG was more effective than *bevacizumab* and b-PLGA.

Conclusion: The reduction in CNV area in all treatment subgroups, with the exception of those in group 3, suggests successful creation of the 2 *bevacizumab* formulations while retaining its active antiangiogenic properties. Further studies varying in dosages and timing of injection and laser are needed to evaluate the formulations' long-acting efficacy.

Introduction

AGE-RELATED MACULAR degeneration (AMD) is the leading cause of legal blindness in patients over age 50 in developed western countries.^{1–3} Although both forms of AMD (dry and wet) affect central vision, the wet or exudative form poses the greatest risk for severe visual loss. The wet form is characterized by choroidal neovascularization (CNV); thus, the majority of treatments have been geared toward the wet form and the treatment of CNV.

Anti-vascular endothelial growth factor (VEGF) agents have emerged as a key therapeutic drug class for treating neovascular diseases of the eye.^{4–6} Prior therapies included

laser photocoagulation, photodynamic therapy, and steroids. *Pegaptanib sodium* (Macugen; Eyetech, Inc., New York, NY) was the first Food and Drug Administration (FDA) approved anti-VEGF treatment for wet AMD.⁷ *Ranibizumab* (Lucentis; Genentech, Inc., South San Francisco, CA) received FDA approval 2 years later. The Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular AMD (MARINA) and Anti-VEGF Antibody for the Treatment of Predominantly Classic CNV in AMD (ANCHOR) studies established *ranibizumab* as the superior treatment for wet AMD, compared with any prior FDA-approved treatments.^{8,9} *Bevacizumab* (Avastin[®]; Genentech, Inc.), a recombinant humanized monoclonal immunoglobulin

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antibody, is an anti-VEGF agent that received FDA approval as an adjunct treatment of colorectal cancer.¹⁰ Philip Rosenfeld of the University of Miami pioneered the use of *bevacizumab* in the treatment of eye diseases, after early data using *bevacizumab* intravenously suggested its efficacy in treatment of wet AMD.^{11,12} The off-label use of intravitreal *bevacizumab* has since become a mainstay of treatment of wet macular degeneration worldwide.¹³ Studies have also shown that *bevacizumab* is effective at decreasing neovascularization in the anterior segment of the eye and as a treatment of neovascular glaucoma.^{5,6,14}

The large-scale, randomized clinical trials, such as MARINA and ANCHOR studies, which support the use of anti-VEGF therapy for wet AMD, are all based on monthly intravitreal anti-VEGF injections for 2 years. One of the current clinical challenges is to determine a regimen with reduced frequency without compromising visual acuity outcomes. The PIER study tested a reduced-frequency, fixed-dosing regimen of ranibizumab of 3 initial monthly injections followed by single injections every 3 months.¹⁵ The SUSTAIN study drew on OCT results to guide retreatment decisions. A 2-year open label continuation study of ANCHOR and MARINA, known as HORIZON, utilized the clinical judgment of investigators and whatever imaging the investigator judged was appropriate to direct retreatment decisions. In all 3 studies, reduced-frequency treatments reduced substantial visual acuity loss; however, the chance of substantial visual acuity gain seemed less likely than with the monthly treatments in the original MARINA and ANCHOR studies.^{9,16} The Prospective OCT Imaging of Patients with Neovascular AMD Treated with intraOcular Ranibizumab (PrONTO) study was a 2-year prospective, open-label, single-center trial with OCT-guided variable-dosing regimen based at the University of Miami. They reported comparable visual acuity outcomes from the phase III clinical studies (MARINA and ANCHOR) but required fewer intravitreal injections.¹⁷

Attention has also been turned toward extended-release or sustained-release anti-VEGF agents as well as implantable devices for long-term drug release.^{18,19} In this study, we prepared 2 *bevacizumab* formulations and studied their effects on the reduction of experimentally induced CNV in rat eyes. Dosing regimens differing in sequence and time as described in Table 1 were used to determine the long-acting potential of these compounds.

Methods

Formulation of Bevacizumab compounds

An aliquot of 30 μ L *bevacizumab* stock solution (25 mg/mL) was diluted with 60 μ L of dilution buffer. This resulted in a final concentration of 12.5 mg/mL of *bevacizumab*. Two additional *bevacizumab* preparations were formulated—a pegylated *bevacizumab* and a poly(lactic-co-glycolic acid) (PLGA)-encapsulated *bevacizumab* (b-PLGA). A carboxyl-group-terminated poly(ethylene-glycol) (PEG) (molecular weight: 10,000) was conjugated to *bevacizumab* by carbodiimide-mediated coupling reaction in the molar ratio of 1:1, resulting in 0.95 mg of *bevacizumab* per mg of conjugate. About 1.05 mg of PEG-*bevacizumab* conjugate (b-PEG) was dispersed in 120 μ L of dilution buffer, with a final *bevacizumab* concentration of 8.313 mg/mL. *Bevacizumab*-loaded nanoparticles were prepared by a solid-in-oil-in-water en-

TABLE 1. TREATMENT GROUPS BASED ON INJECTION SCHEDULE

	Week 0	Week 2	Week 4	Week 6	Week 8
Group 1	Laser	Injection	Harvest		
Group 2	Injection	Laser	Harvest		
Group 3	Injection		Laser	Harvest	
Group 4	Injection			Laser	Harvest

capsulation method using PLGA polymer (Resomer RG 503 H; inherent viscosity (i.v.) 0.63 dL/g; molecular weight: 38,000–54,000), with a final drug load of 22 μ g of *bevacizumab* per mg of nanoparticle (mean particle size of *bevacizumab*-loaded nanoparticle of 819 nm). One hundred thirty-six milligrams of b-PLGA was dispersed in 360 μ L of dilution buffer, resulting in a final *bevacizumab* concentration of 8.311 mg/mL.

Animal selection and photocoagulation

All animal research adhered to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Twenty-four male adult Brown Norway rats received intraperitoneal injections of ketamine (60 mg/kg) and xylazine (10 mg/kg) to induce anesthesia. Subsequently, 1% tropicamide was applied topically to dilate both pupils. The photocoagulation technique employed was similar to that described by Edelman and Castro.²⁰ Using a glass coverslip over the eye as a lens, 6 laser spots were placed in the peripapillary area of each eye, $\sim$ 300 μ m apart, using a slit lamp/argon green laser apparatus (Lumenis Inc., Santa Clara, CA), to deliver laser spots of 200 mW power, 50- μ m spot diameter, and 100 ms duration.

Intravitreal injection

In each group of 12 eyes of 6 rats, 3 eyes were assigned to a treatment subgroup, each receiving a different injection—control (sodium citrate), *bevacizumab*, b-PEG, and b-PLGA. Ofloxacin was applied topically immediately before and after each injection, and 5% betadine solution was applied similarly before any manipulation. Intravitreal injections were performed using a method similar to that described by Gao et al.²¹ A volume of 5 μ L was injected intravitreally using a Hamilton microinjector (Hamilton Co., Reno, NV). This resulted in a total dose of 62.5 μ g of *bevacizumab* and 41.6 μ g of b-PEG and b-PLGA. Direct visualization with the slit lamp was used to confirm proper placement. Topical ofloxacin was applied daily for 1 week following treatment.

Laser photocoagulation and injection schedule

The 48 eyes from the 24 rats were divided equally into 4 groups. In all eyes, laser photocoagulation was used to rupture Bruch's membrane as described earlier. In group 1, laser was followed by injection, which was then followed by harvesting the rats to assess the CNV area. All 3 steps were separated by a 2-week interval. In groups 2, 3, and 4, injection preceded laser photocoagulation by a variable interval and all rats were harvested 2 weeks postlaser treatment. In group 2, laser and injection were separated by 2 weeks. In group 3, laser followed injection by 4 weeks. In group 4, laser followed injection by 6 weeks (Table 1).

Fluorescein isothiocyanate–dextran angiography and flat-mount preparation

At the scheduled harvest date for each treatment group, the animals were again anesthetized with ketamine 80 mg/kg and xylazine 10 mg/kg. The perfusion of retinal vasculature with fluorescein isothiocyanate (FITC)–dextran and the preparation of flat mounts were both performed as described by McMenamin²² and Semkova et al.²³ An incision was made in the left ventricle and a cannula was inserted, and the descending aorta was then occluded with a clamp. A constant flow pump (Harvard Apparatus, Holliston, MA) was utilized to perfuse the vasculature. Each animal received an infusion of 120 mL of cold heparinized (1 IU/mL) phosphate-buffered saline followed by infusion of 250 mg of 2-million-molecular-weight FITC–dextran (Sigma-Aldrich Inc., St. Louis, MO) in a volume of 50 mL of phosphate-buffered saline. The right atrium was also incised to allow efflux of the perfusate. The animals were euthanized with 100% CO₂ and the eyes were enucleated and placed in 10% formalin solution overnight. The posterior cup was dissected from the anterior portion and vitreous at the equator. Carefully, the neural retina was removed, and in the remaining RPE-choroid-sclera, 4 radial cuts were placed, allowing the tissue to flatten easily. The sections were then flat-mounted on standard microscope slides, treated with epifluorescent microscopy-specific medium (Biomed Corporation, Foster City, CA), and then further compressed with weights placed on top of the coverslips. Fingernail polish was applied to the perimeter of the coverslips to prevent tissue desiccation.

Quantification of CNV area and statistical analysis

The techniques of Edelman and Castro were used to visualize and quantify the FITC–dextran-labeled CNV.²⁰ Using

the appropriate FITC filters, the flat mounts were viewed under the 20× objective of an epifluorescent microscope. A personal computer-operated digital camera with image capture and analysis software was used to photograph the areas of CNV. To quantify the FITC–dextran-perfused vessels representing CNV at the site of laser trauma, the hyperfluorescent pixels in the area of the laser burn were delineated. Bridging neovascularization was included by measuring the total area of the bridging CNV membrane and then dividing by the number of underlying laser lesions from which the network originated. One-way analysis of variance was conducted to analyze the effect of the intravitreal injections on CNV area. In addition, pairwise comparisons were conducted to assess for statistically significant differences between each treatment subgroup with the control.

Results

With 6 laser spots per eye and 3 eyes per treatment subgroup, a maximum of 18 CNV areas were possible. Number of CNV areas ranged from 11 to 16 in our study. The mean CNV areas of the treatment subgroups, as well as the percentage of decrease in CNV area from the control for each subgroup, are listed in Table 2. In group 1, a decrease of CNV area from control of 45.9%, 40.4%, and 32.7% was found for *bevacizumab*, b-PEG, and b-PLGA, respectively. All mean CNV areas measured in pixels were found to be statistically significant, compared with control ($P < 0.001$) (Fig. 1). In group 2, there was also a statistically significant decrease in CNV area from the control for all 3 treatment groups. *Bevacizumab* decreased CNV area by 38.0% ($P < 0.001$), b-PEG decreased CNV area by 40.9% ($P < 0.001$), and b-PLGA decreased CNV by 34.8% ($P = 0.005$) (Fig. 2). In group 3, there was no statistically significant difference between the mean CNV area in the *bevacizumab* treatment

TABLE 2. PERCENTAGE OF DECREASE IN CHOROIDAL NEOVASCULARIZATION AREA IN ALL GROUPS

Treatment group	Injection	Number of CNVs measured	Mean CNV area (st. dev.)	% decrease from control	P value
Group 1	Control	12	25,473 (5,684)		
	Bevacizumab	16	13,790 (4,114)	45.9	<0.001
	b-PEG	15	15,186 (4,829)	40.4	<0.001
	b-PLGA	14	17,142 (3,581)	32.7	<0.001
Group 2	Control	13	26,606 (4,899)		
	Bevacizumab	12	16,490 (3,916)	38.0	<0.001
	b-PEG	13	15,737 (2,661)	40.9	<0.001
	b-PLGA	11	17,347 (8,032)	34.8	0.005
Group 3	Control	16	15,932 (5,691)		
	Bevacizumab	14	17,377 (6,587)	No decrease in subgroup	0.529
	b-PEG	12	14,491 (2,705)	9.0	0.384
	b-PLGA	12	12,693 (3,560)	20.3	0.077
Group 4	Control	14	16,003 (2,367)		
	Bevacizumab	11	13,022 (2,123)	18.6	0.003
	b-PEG	13	10,196 (3,133)	36.3	<0.001
	b-PLGA	14	13,360 (2,917)	16.5	0.015

b-PEG, poly(ethylene-glycol)-*bevacizumab* conjugate; b-PLGA, poly(lactic-co-glycolic acid)-encapsulated *bevacizumab*; CNV, choroidal neovascularization; st. dev., standard deviation.

FIG. 1. Group 1: Average choroidal neovascularization (CNV) area interval plot.

subgroup compared with the control subgroup ($P=0.529$) (Fig. 3). Both extended release formulations showed a decrease in CNV area when compared with control, 9.0% for b-PEG and 20.4% for b-PLGA; however, neither of these was found to be statistically significant ($P=0.384$ and $P=0.077$, respectively). In group 4, there was an 18.6% ($P=0.003$) decrease in CNV area in the *bevacizumab* subgroup, 36.3% ($P<0.001$) for b-PEG, and 16.5% ($P=0.015$) for b-PLGA (Fig. 4).

Discussion

The use of polymer encapsulation and pegylation of drugs to produce long-lasting drug concentrations has received considerable attention over the past few years. Composite biodegradable polymeric particles entail a drug that is surrounded by or dispersed in a polymer matrix. The polymers can consist of either natural or synthetic materials. Cross-linking in the microencapsulation process is necessary with the use of natural materials. This leads to the denaturation of the polymer and the embedded drug²⁴; therefore, synthetic polymers are more commonly used. Poly(α -hydroxy) acids, including PLGA, are frequently used synthetic polymers. The release of the active drug from the polymer matrix is dependent on diffusion through channels and pores in the polymer matrix and across the polymer barrier and on polymer degradation.²⁴ The controlled delivery of drugs

FIG. 3. Group 3: Average CNV area interval plot.

with polymers as implants, microspheres, and nanoparticles have gained wide acceptance. An *in vivo* study in rabbits investigated nanosuspension formulations of glucocorticoid eyedrops and revealed enhanced ocular absorption and sustained drug release with increased effect over a longer duration.²⁵ Another study looked at an intravitreal injection of dexamethasone acetate encapsulated in PLGA and its effect on CNV. This study demonstrated a triphasic pattern of drug release, corresponding to the initial release of the drug adsorbed onto the outer particle surface, a second slow release through diffusion of the drug out of the matrix, and a final third phase caused by the polymer degradation.²⁶

Modification of drug molecules with PEG is a well-developed process aimed toward improving the delivery of injectable drugs and reducing adverse effects.²⁷ The process of pegylation may alter the drug molecule, resulting in enhanced pharmacokinetic and pharmacodynamic properties for PEG-modified proteins compared with unmodified proteins. A direct relationship between the mass of the PEG, circulating half-life, and area under the curve of the therapeutic molecule has been demonstrated.²⁷ However, an excessively large PEG molecule can decrease the bioactivity of the drug because of possible blockage or PEG attachment to the receptor-binding sites.²⁷ Current systemic pegylated agents that are commercially available include PEG-adenosine deaminase (ADA) for use in the treatment of ADA deficiency in patients with severe combined immunodeficiency.

FIG. 2. Group 2: Average CNV area interval plot.

FIG. 4. Group 4: Average CNV area interval plot.

FIG. 5. (A) CNV area for control-treated eye. (B) CNV area for poly(lactic-co-glycolic acid)-encapsulated bevacizumab-treated eye.

ciency disease and pegylated asparaginase for the treatment of acute lymphoblastic leukemia. In both agents, the pegylation produced a less-immunogenic molecule resulting in increased stability and an extended half-life.^{27–29}

With the exception of group 3, all treatment subgroups showed a statistically significant decrease in CNV area relative to saline-injected controls. This suggests successful formulation of PEG-bevacizumab conjugate and b-PLGA while retaining the active antiangiogenic properties (Fig. 5). However, there are limitations to our study.

Bevacizumab is a humanized monoclonal antibody to VEGF. Humanization of monoclonal antibodies is performed to decrease the immunogenicity of antibodies generated in a nonhuman immune system when administered in a human patient.³⁰ There is much debate regarding the efficacy of bevacizumab in nonprimate species, including rats. Manzano et al. reported decreased corneal neovascularization with topically administered bevacizumab in a rat model.³¹ However, other studies have suggested that bevacizumab does not inhibit leakage of laser-induced CNV in rats and the response to anti-VEGF therapy may be species and site specific.³²

Assuming that bevacizumab reduces CNV in rats, the inability to demonstrate the long-acting effects of the bevacizumab formulations compared with control may be due to several reasons. First, the rats may have been harvested prematurely, not allowing sufficient time to lapse and demonstrate extended anti-VEGF activity of the b-PEG conjugate and b-PLGA nanoparticles. Longer time intervals were precluded by corneal irritation and subsequent scarring from the laser and injections. Second, the bevacizumab-alone injection had a higher concentration than the b-PEG and b-PLGA injections, 12.5 versus 8.313 mg/mL and 8.311 mg/mL, respectively. Third, assuming a volume of 4 mL for the vitreous cavity in humans, the normal bevacizumab injection for wet AMD of 1.25 mg/0.05 mL would result in a concentration of 0.31 mg/mL. In our study, assuming a rat vitreous volume of 13.36 μ L,³³ the intravitreal concentration of bevacizumab-alone, b-PEG, and b-PLGA were 4.68, 3.11, and 3.11 mg/mL, respectively. This far exceeds the usual dosage in humans. In addition, there were only 3 eyes in each treatment subgroup. Further, the polymeric systems release drug in a slower fashion unlike bevacizumab alone, which provides the entire drug in free form at time zero. Though the reduction in CNV area compared to control was found to be statistically

significant in 3 of the treatment groups, there existed a large variability in CNV area.

It remains unclear whether bevacizumab has anti-VEGF properties in a rat model. However, our study certainly suggests that bevacizumab may decrease experimentally induced CNV in rats. Additional studies with a larger number of eyes per subgroup and lower dosages of intravitreal injections are warranted to study the long-acting potential in both the pegylated bevacizumab conjugate and b-PLGA nanoparticles.

Acknowledgments

The authors are thankful to Dr. Swita Singh for the preparation of dosing solutions and assistance during animal dosing with various formulations. Support: NIH EY017045 (UBK through Emory University).

Disclosure Statement

The authors have no competing financial interests to disclose.

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Received: November 6, 2010

Accepted: April 13, 2011

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