Suppression and Regression of Choroidal Neovascularization by Polyamine Analogues

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PURPOSE. Polyamine analogues inhibit tumor growth in vitro and in vivo, and oligoamines with a chain length of 10, 12, or 14 are particularly potent. This study was conducted to investigate the effect of the decamers CGC-11144 and CGC-11150 in a mouse model of choroidal neovascularization (CNV).

METHODS. Mice with laser-induced rupture of Bruch’s membrane were given intraperitoneal, intravitreal, or periocular injection of CGC-11144, CGC-11150, or vehicle, and after 14 days, they were perfused with fluorescein-labeled dextran, and the area of CNV was measured on choroidal flatmounts by image analysis. In some groups of mice, treatments were started 7 days after rupture of Bruch’s membrane to determine the effect of the agent on established CNV. Electroretinograms (ERGs) were performed to assess the effects on retinal function, and histopathology was used to evaluate retinal structure.

RESULTS. Intraperitoneal injection of 10 or 20 mg/kg CGC-11144 or CGC-11150 resulted in small but significant reductions in the area of CNV. Intravitreal injection of 20 μg CGC-11144 or CGC-11150 on days 0 and 7 after rupture of Bruch’s membrane resulted in a ~40% reduction in the area of CNV, with a similar reduction after periocular injections of 0.2 mg CGC-11144 three times a week for 2 weeks. Both intravitreal and periocular delivery of CGC-11144 also caused significant regression of established CNV. Within 2 days of periocular injection of CGC-11144, there was apoptosis in CNV lesions, but not in normal blood vessels or other retinal cells. Periocular injections of D,L-α-difluoromethyl-ornithine (DFMO), which decreases polyamine levels by a different mechanism, also inhibited CNV. There was no decline in ERG amplitudes or abnormal retinal morphology after daily injections of 0.2 mg CGC-11144 for 2 weeks, but a single intravitreal injection compromised retinal structure and function.

CONCLUSIONS. Periocular delivery of the polyamine analogues may be a useful approach for the treatment of CNV. (Invest Ophthalmol Vis Sci. 2005;46:3323–3330) DOI:10.1167/iovs.04-1210

The polyamines, putrescine, spermidine, and spermine, are necessary for cell growth and are present at high levels in rapidly proliferating tissues.1–3 It has been hypothesized that blockade of polyamine synthesis can provide a treatment approach for cancer and other diseases in which cell proliferation plays a major role. The development of D,L-α-difluoromethyl-ornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), a critical enzyme in the polyamine biosynthetic pathway, provides a tool to test this hypothesis.4 In several mouse tumor models, DFMO was well tolerated and inhibited tumor growth, raising enthusiasm for clinical trials.5 Although beneficial effects were found in patients with recurrent glialoma,6 trials investigating effects in patients with other types of tumors showed little or no effect.7 Part of the problem stems from multiple feedback loops that maintain intracellular polyamine levels within a narrow range. Low levels stimulate biosynthesis and transport and reduce degradation. High polyamine levels, by a very unusual mechanism, induce translation of antizyme, a 26-kDa protein that blocks polyamine transport and ODC activity, and also promotes degradation of ODC. Blocking polyamine biosynthesis with DFMO results in upregulation of polyamine transport and recovery of depleted intracellular levels from extracellular stores.8,9 The experience with DFMO supported targeting polyamines in the treatment of proliferative diseases, but showed that it would be more difficult to achieve than initially thought.

Another strategy was to take advantage of the ability of polyamines to regulate their own levels. Synthesis was achieved of polyamine analogues that are sufficiently similar to native polyamines to enter cells through the polyamine transporter and provide feedback inhibition of polyamine synthesis and transport, but are different enough from native polyamines that they do not promote proliferation and survival.10 Whether this is actually the mechanism of action or not, several polyamine analogues have been shown to inhibit growth of tumor cells in vitro and in vivo. A series of oligoamines have been shown to be particularly potent antitumor agents.11

Since in neovascular disorders there is excessive proliferation of vascular cells, it is reasonable to hypothesize that polyamine analogues can be useful in their treatment. In support of this possibility, spermidine and spermine stimulate angiogenesis in chick chorioallantoic membranes (CAMs),12 and DFMO inhibits the angiogenesis surrounding B16 melanoma implanted in CAMs.13 However, results in one vascular bed do not necessarily predict results in others.14 In this study,
we sought to determine whether the oligoamines CGC-11144 and CGC-11150 inhibit choroidal neovascularization (CNV).

**MATERIALS AND METHODS**

**Drugs**

The decamines CGC-11144 and CGC-11150 (formerly SL-11144 and SL-11150), were synthesized as previously described.\(^{11,15}\) The agents were diluted in phosphate-buffered saline for injections. DFMO was obtained from Sigma-Aldrich (St. Louis, MO).

**Preventive Treatment of Laser-Induced CNV**

Mice were treated in accordance with the recommendations of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Laser-photocoagulation-induced rupture of Bruch's membrane was used to generate CNV.\(^{16}\) Briefly, 4- to 5-week-old female C57BL/6J mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight), and the pupils were dilated with 1% tropicamide (Alcon Laboratories, Inc., Forth Worth, TX). Three burns of 532-nm diode laser photocoagulation (75-μm spot size, 0.1-second duration, 120 mW) were delivered to each retina by using the slit lamp delivery system of a photocoagulator (OcuLight; Iridex, Mountain View, CA) and a handheld coverslip as a contact lens. Burns were performed in the 9, 12, and 3 o'clock positions of the posterior pole of the retina. Production of a bubble at the time of lasering, which indicates rupture of Bruch's membrane, is an important factor in obtaining CNV,\(^{16}\) and so only burns in which a bubble was produced were included in the study.

Four independent experiments were performed to investigate the effect of intraperitoneal (IP) injections: (1) 8 mice received twice-a-week IP injections of 10 mg/kg CGC-11144, and 8 mice received twice-a-week IP injections of vehicle; (2) 10 mice received three times-a-week IP injections of 20 mg/kg CGC-11144, and 8 mice received three times-a-week IP injections of vehicle; (3) 8 mice received twice a week IP injections of 10 mg/kg CGC-11150, and 8 mice received twice a week IP injections of vehicle; and (4) 10 mice received three times-a-week IP injections of 5 mg/kg CGC-11150, and 9 mice received three times a week IP injections of vehicle. Two independent experiments were performed to investigate the effect of intravitreal injections: (1) 10 mice received an injection of 20 μg CGC-11144 in one eye and vehicle in the fellow eye on days 0 and 7 after rupture of Bruch's membrane,\(^{2}\) 10 mice received an injection of 20 μg CGC-11150 in one eye and vehicle in the fellow eye on days 0 and 7 after rupture of Bruch's membrane. Experiments were performed to investigate the effect of periorcular injection of CGC-11144 in which 10 mice received a periorcular injection three times a week of 0.2 mg CGC-11144 in 5 μL of PBS in one eye and 5 μL of PBS in the fellow eye. Five mice received no treatment and served as the control for effects of systemic absorption in the fellow eye.

Two weeks after rupture of Bruch's membrane, mice were anesthetized and perfused with fluorescein-labeled dextran (2 × 10⁶ average molecular weight, Sigma-Aldrich) and choroidal flatmounts were prepared as previously described.\(^{17}\) Briefly, the eyes were removed, fixed for 1 hour in 10% phosphate-buffered formalin, and the cornea and lens were removed. The entire retina was carefully dissected from the eyecup, radial cuts were made from the edge of the eyecup to the equator in all four quadrants, and it was flattened in aqueous mounting medium (Aquamount; BDH, Poole, UK). Flatmounts were examined by fluorescence microscopy (Axioskop; Carl Zeiss Meditec, Thornwood, NY), and images were digitized with a three charge-coupled device (CCD) color video camera (IK-TU40A; Toshiba, Tokyo, Japan) and a frame grabber. Image-analysis software (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) was used to measure the area of each CNV lesion. Statistical comparisons were made in a linear mixed model.\(^{18}\) This model is analogous to analysis of variance (ANOVA), but allows analysis of all CNV area measurements from each mouse, rather than average CNV area per mouse, by accounting for correlation between measurements from the same mouse. The advantage of this model over ANOVA is that it accounts for differing precision in mouse-specific average measurements arising from a varying number of observations among mice. Probabilities for comparison of treatments were adjusted for multiple comparisons by the Dunnett method.

**Treatment of Established CNV**

Adult female C57BL/6 mice had laser treatment to three locations in each eye as described earlier. Only burns in which a bubble was produced were included. After 1 week, some mice were used to measure the baseline amount of CNV present at 7 days. Other mice were treated with 20 μg CGC-11144 in one eye and vehicle in the fellow eye on days 7 and 10, or they were treated with periorcular injections of 200 μg CGC-11144 in one eye and vehicle in the fellow eye on days 7, 10, and 13. On day 14, the mice were perfused with fluorescein-labeled dextran and the CNV area was measured at Bruch's membrane rupture sites on choroidal flatmounts. In some experiments, mice underwent the rupture of Bruch’s membrane and then between days 7 and 14 were given daily periorcular injections of 5 μL vehicle in one eye and 5 μL containing 100 μg of DFMO, 200 μg CGC-11144, or a combination of 100 μg of DFMO and 200 μg CGC-11144 in the fellow eye.

**Recording and Analysis of ERGs**

Adult female C57BL/6 mice were given an intravitreal injection of 2, 4, or 20 μg CGC-11144 in one eye and an injection of vehicle in the fellow eye and, after 3 days, ERGs were recorded as previously described.\(^{19}\) Mice were dark adapted for a standardized 12-hour period overnight, and ERG recordings were performed (Espion ERG; Diagnosys LLI, Littleton, MA). All manipulations were performed with dim red light illumination. Beginning the same time each morning, mice were anesthetized by IP injection of 25 μL/g body weight of tri bromoethanol (Avertin; Aldrich, Milwaukee, WI) diluted 1:39 in PBS. Corneas were anesthetized with a drop of 0.5% proparacaine hydrochloride (Alcon Laboratories), and pupils were dilated with 1% tropicamide. Mice were placed on a pad heated to 39°C, and platinum electrodes were placed on each cornea after application of a gonioscopic prism solution (Alcon Laboratories). The reference electrode was placed subcutaneously in the anterior scalp between the eyes, and the ground electrode was inserted into the tail. Electrode impedance was balanced for each eye pair measured. The head of the mouse was placed in a standard position in a Ganzfeld bowl illuminator that assured equal illumination of the eyes. Simultaneous recordings from both eyes were made at 11 intensity levels of white light ranging from −3.00 to +1.40 log cd−s/m². The ERG machine (Espion; Diagnosys) measures the response six times at each flash intensity and records the average value. ERGs were also performed after daily periorcular injections of 0.2 mg CGC-11144 for 2 weeks.

**TUNEL Staining**

On days 7 and 8 after laser-induced rupture of Bruch’s membrane, adult female C57BL/6 mice received a periorcular injection of 0.2 mg CGC-11144 or vehicle and then were euthanatized. Eyes were embedded in optimal cutting temperature compound (OCT) and 10-μm serial sections were cut through CNV lesions and fixed with 1% paraformaldehyde for 10 minutes at room temperature. TUNEL staining was performed with a kit (ApopTag Fluorescein Red; Intergen, Purchase, NY) according to the manufacturer’s instructions. Adjacent sections were stained with *Griffonia simplicifolia* agglutinin (GSA), as described.\(^{17}\)
RESULTS

Effect of Systemic Administration of Polyamine Analogue on CNV at Bruch’s Membrane Rupture Sites

IP injections of 10 mg/kg CGC-11144 (Fig. 1A) or CGC-11150 (Fig. 1C) twice a week were well tolerated and caused small, but statistically significant reductions in the size of CNV lesions. Injections of 20 mg/kg CGC-11144 (Fig. 1B) or CGC-11150 (Fig. 1D) IP twice a week were not well tolerated, and several mice died during the 2-week treatment period. Mice that survived showed small statistically significant reductions in CNV lesion size compared with vehicle-injected control animals. The reductions were comparable to the reductions recorded at the 10-mg/kg doses. Since 20 mg/kg was above the maximum tolerated dose, further increases in dose would not be useful, and therefore other routes of delivery were considered.

Effect of Intravitreous or Periocular Injections of Polyamine Analogue on Reduction of CNV at Bruch’s Membrane Rupture Sites

Eyes that received no treatment had consistent amounts of CNV 14 days after rupture of Bruch’s membrane (Figs. 2A, 2D). Other mice received intravitreous injection of 20 μg CGC-11144 or CGC-11150 immediately after laser-induced rupture of Bruch’s membrane and 7 days after laser treatment in one eye and vehicle injections in the fellow eye. In eyes that received intravitreous injections of vehicle, the area of CNV at Bruch’s membrane rupture sites (Figs. 2B, 2E) looked very similar to that in untreated eyes (Figs. 2A, 2D). In contrast, the size of CNV lesions at rupture sites appeared smaller in eyes treated with CGC-11144 (Fig. 2C) or CGC-11150 (Fig. 2F). Measurement of CNV area by image analysis showed that eyes injected with CGC-11144 (Fig. 2G) or CGC-11150 (Fig. 2H) had a statistically significant decrease in area of CNV by approximately 40% compared to vehicle-treated eyes or untreated eyes. The lack of a difference between untreated eyes and fellow eyes treated with vehicle suggests that there was no systemic effect from intraocular injection of CGC-11144 or CGC-11150.

Effect of Intravitreous Injection of Polyamine Analogue on Established CNV

To determine whether polyamine analogues have any effect on established CNV, mice had rupture of Bruch’s membrane at three locations in each eye and were divided into two groups. In one group, mice were perfused 7 days after rupture of Bruch’s membrane, and the area of CNV at rupture sites was measured on choroidal flatmounts. The remainder of the mice received intravitreous injections of 20 μg CGC-11144 in one eye and vehicle in the other eye on days 7 and 10. At 14 days after rupture of Bruch’s membrane, eyes that had been injected with CGC-11144 had CNV lesions that were significantly smaller in area than those present in fellow eyes that had been treated with vehicle, and also significantly smaller than the area received in untreated eyes.
of CNV lesions in the 7 day baseline eyes, indicating that there had been regression of CNV (Fig. 3A).

**Effect of Intravitreous Injection of CGC-11144 on Retinal Structure and Function**

The ERG provided an assessment of retinal functioning. Three days after intravitreous injection of 20 μg CGC-11144 both ERG a- and b-wave amplitudes were dramatically reduced (Fig. 3B). Injection of 10 μg CGC-11144 also caused a striking decrease in ERG amplitudes, and whereas a-wave amplitudes were only mildly decreased at highest flash intensities after injection of 2 μg CGC-11144, b-wave amplitudes were profoundly reduced at all flash intensities. Retinal sections from eyes injected with 20 μg CGC-11144 illustrate that retinal structure, as well as function, was markedly disrupted (Figs. 3C, 3D), compared with fellow eyes injected with vehicle (Figs. 3E, 3F). An intravitreous injection of 20 μg CGC-11150 also caused retinal damage (not shown).

**Effect of Periocular Injection of CGC-11144 on CNV and Retinal Function**

Many drugs and even proteins injected into the periocular space adjacent to the outside of the sclera achieved high levels in the choroid and much lower levels in the retina. Fourteen days after rupture of Bruch’s membrane there were large areas of CNV in eyes that received no treatment (Fig. 4A) or periocular injections of vehicle (Fig. 4B). The CNV at Bruch’s membrane rupture sites appeared smaller in eyes treated three times a week with periocular injections of 200 μg CGC-11144 (Fig. 4C). Measurement of CNV areas by image analysis showed that eyes treated with periocular CGC-11144 had significantly less CNV than corresponding control eyes (no injection or vehicle-injected fellow eye). The bars show the mean (± SEM) area of CNV, calculated from the total number of rupture sites for which measurements were taken in each group (n). †P < 0.0001 for difference from no-treatment group, by linear mixed model with the Dunnett method for multiple comparisons; *P < 0.0001 for difference from vehicle control group (fellow eyes) by linear mixed model with the Dunnett method for multiple comparisons.

**Effect of Periocular Injections of DFMO or CGC-11144 on CNV**

In an attempt to increase the inhibitory effect, two strategies were tried. One was to increase the frequency of injections of CGC-11144 and the other was to use CGC-11144 in combination with another drug that antagonizes polyamines by a different mechanism. Like polyamine analogues, DFMO reduces...
intracellular polyamine levels, but it acts by blocking synthesis rather than acting as a polyamine mimic with altered function, as is the case with polyamine analogues. Daily periocular injections of 5 μL containing 100 μg DFMO between days 7 and 14 resulted in a significant reduction in the area of CNV at Bruch’s membrane rupture sites compared with the baseline amount present at day 7 (Fig. 5). Daily periocular injections of 5 μL containing 200 μg CGC-11144 between days 7 and 14 resulted in significant reduction in CNV area very similar to that obtained with daily injections of 100 μg DFMO. Coinjection of 200 μg CGC-11144 and 100 μg DFMO did not result in further enhancement of the regressive effect of either alone.

FIGURE 5. Intravitreous injection of CGC-11144 also caused regression of established CNV, but disrupted retinal function and structure. (A) Fifteen mice had laser-induced rupture of Bruch’s membrane at three locations in each eye and after 7 days, five mice were perfused with fluorescein-labeled dextran, and the baseline area of CNV was measured. The remaining 10 mice were given an intravitreous injection of 20 μg CGC-11144 in one eye and vehicle in the fellow eye on days 7 and 10, and CNV area was measured at each rupture site at 14 days after injury (n = 30 in each group). The mean area of CNV was significantly smaller in eyes injected with CGC-11144 than in vehicle-injected fellow eyes, or compared with the baseline area of CNV measured at 7 days. This indicates that intravitreous injection of CGC-11144 caused regression of established CNV. ^P < 0.0009 for the difference from the baseline amount of CNV at 7 days, by linear mixed model with the Dunnett method for multiple comparisons. *P < 0.0001 for difference from vehicle-injected fellow eye at 14 days, by linear mixed model with the Dunnett method. (B) Mice were given an intravitreous injection of 2, 4, or 20 μg CGC-11144 in one eye and PBS in the fellow eye. After 3 days, ERGs were performed. Eyes injected with 4 or 20 μg CGC-11144 had a significant decrease in a-wave amplitude, and eyes injected with 4, 20 μg CGC-11144 had a significant decrease in b-wave amplitude, compared with eyes injected with PBS (P < 0.05; ANOVA). (C, D) Two weeks after intravitreous injection of 20 μg CGC-11144, there was substantial disruption of the morphology of the retina, particularly the inner retina. (E, F) Two weeks after intravitreous injection of PBS, the retina had a normal appearance. Retinal sections were stained with hematoxylin and eosin; Magnification: (C, E), ×40; (D, F) ×100.
Effect of Periocular Injection of CGC-11144 on Apoptosis within CNV Lesions

On days 7 and 8 after laser-induced rupture of Bruch’s membrane, mice received a periocular injection of 0.2 mg CGC-11144 or vehicle and then were euthanatized. Ten-micrometer serial sections were cut through CNV lesions, and staining with GSA was performed to visualize vascular cells on some sections and TUNEL staining was performed to identify cells undergoing apoptosis on adjacent sections. Eyes that had received periocular injections of CGC-11144 showed many TUNEL-stained cells within CNV lesions (Figs. 6A, 6B), whereas there was no detectable apoptosis in CNV lesions from eyes injected with vehicle (Figs. 6C, 6D).

DISCUSSION

Several lines of evidence have implicated vascular endothelial growth factor (VEGF) as a critical stimulus for ocular NV. Expression of VEGF correlates temporally and spatially with retinal NV and signaling through VEGF receptors is both necessary and sufficient for development of retinal NV. VEGF is also a major stimulus of CNV. These data suggest that VEGF may be an important therapeutic target, and a large clinical trial and smaller trials or case series support this contention (Rosenfeld PJ, et al. IOVS 2003;44:ARVO E-Abstract 970).

Although the level of benefit that is achievable with various agents that block VEGF or VEGF receptors is not yet known and must be explored in detail, it is also important to develop a long-term strategy to maximize the effects of antiangiogenic drug therapy. A reasonable approach is to identify other drugs that inhibit ocular NV by different mechanisms. Such drugs may exhibit additive effects when combined with VEGF antagonists.

In this study, specific polyamine analogues suppressed the development and promoted regression of CNV. Although some biological activity was achieved with systemic administration of CGC-11144 and CGC-11150, it was slight, and it is unlikely that systemic administration is a viable clinical approach. However, local administration by either intravitreous or periocular injections not only caused suppression, but also induced regression of established CNV. Over a 7-day treatment period, periocular injections not only caused suppression, but also induced regression of established CNV.
The regression was not complete and could not be increased beyond 40% by increasing the dose of polyamine analogues or by combining them with DFMO, an inhibitor of polyamine biosynthesis. To provide perspective on this effect, intravitreal injection of adenoviral vectors expressing pigment epithelium-derived factor caused a similar amount of regression in the same model. Over both a 10-day and a 7-day period, combretastatin-A-4-phosphate, a vascular targeting agent, caused 66% regression of CNV. It is not clear whether more prolonged treatment with polyamine analogues would result in greater regression, but based on the substantial effect over a brief treatment period, local administration of polyamine analogues deserves consideration as a treatment strategy for CNV.

The mechanism by which polyamine analogues exert their antiangiogenic effect is likely to be different from the mechanism by which VEGF antagonists operate. One possible effect of CGC-11144 and CGC-11150 is to induce antizyme, which reduces synthesis and transport of natural polyamines, causing inhibition of proliferation. In addition to halting proliferation, incubation of CGC-11144 or CGC-11150 with breast cancer cell lines in vitro induces apoptosis. Modulation of polyamine metabolism has been demonstrated to induce apoptosis in some situations and prevent it in others. We found that periocular doses that caused regression of CNV caused apoptosis within CNV lesions; therefore, at appropriate doses, polyamine analogues induce apoptosis in cells participating in CNV, with no detectable apoptosis in other vascular cells or neurons in the eye. However, it is clear that this selectivity is relative, because high concentrations in the retina that occur after intravitreal injections result in retinal damage. Since CGC-11144 and CGC-11150 may induce cell death by multiple mechanisms, and some may apply to cells that are not proliferating, it is necessary to be vigilant for toxicity. Although it is possible that there are intravitreal doses lower than those tested in this study that do not cause retinal damage, but still inhibit CNV, careful assessment of safety would be needed before considering intravitreal administration in patients. Theoretically, sustained delivery of low levels would maximize safety and efficacy and would be the most appealing approach for intravitreal administration.

Periocular injections provide an alternative approach for local delivery of polyamine analogues to the eye, and we found that periocular injections of 200 µg CGC-11144 cause suppres-
sion and regression of CNV very similar to that with intraocular injection of 20 μg CGC-11144, but with no evidence of any deleterious effects on retinal morphology or ERG function. Periocular injection of DFMO, which blocks polyamine biosynthesis, also caused partial regression of CNV, and one might anticipate that since DFMO and polyamine analogues perturb polyamine function by different mechanisms, additive inhibition of CNV would be achieved by combining them. However, even a daily periocular dose of 200 μg CGC-11144 combined with 100 μg DFMO did not appear to increase benefit beyond treatment with 200 μg CGC-11144 injected three times a week. Therefore, we may have identified the maximum effect that can be achieved through inhibition of polyamine metabolism, at least over a 1-week period, but that effect was quite impressive. Additional studies are needed to explore further the potential role of periocular delivery of polyamine analogues for treatment of CNV.

References