Safety and Efficacy of Intravitreal Injection of Ranibizumab in Combination With Verteporfin PDT on Experimental Choroidal Neovascularization in the Monkey

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Objective: To study the safety and efficacy of intravitreal injections of anti–vascular endothelial growth factor antibody fragment (ranibizumab [formerly known as rhuFabV2], Lucentis; Genentech, South San Francisco, Calif) in combination with intravenous verteporfin (Visudyne; Novartis, East Hanover, NJ) photodynamic therapy (PDT) on experimental choroidal neovascularization in the monkey eye.

Methods: Choroidal neovascularization was induced by laser injury in both eyes of cynomolgus monkeys and followed with weekly fundus photography and fluorescein angiography. Two weeks after induction, weekly treatments were initiated. These treatments included using either an intravitreal injection of ranibizumab (previously known as rhuFabV2) in combination with verteporfin PDT or a ranibizumab vehicle (placebo) in combination with verteporfin PDT (PDT only). Six animals (group 1) initially received intravitreal injections followed 1 week later by PDT. Four animals (group 2) initially received PDT followed 1 week later by intravitreal injection. Two animals (group 3) received injections and PDT on the same day at 2-week intervals. Photodynamic therapy was applied in all 3 groups every 2 weeks for 3 treatments with follow-up through 2 weeks after the last PDT treatment. Fluorescein angiograms were graded using a masked standardized protocol. The data were analyzed using the McNemar \( \chi^2 \) test for matched pairs.

Results: No choroidal neovascularization leakage was observed in the eyes of animals treated with ranibizumab and PDT at day 21 or 42 after the start of the first treatment. Leakage persisted in eyes treated with PDT alone at 21 days (3 of 12 eyes) and 42 days (2 of 12 eyes). At all time points studied, the ranibizumab and PDT–treated eyes experienced better angiographic outcomes than the eyes receiving PDT alone.

Conclusion: These preliminary data indicate that an intravitreal ranibizumab injection in combination with verteporfin PDT (ranibizumab and PDT) causes a greater reduction in angiographic leakage than PDT and intravitreal vehicle injection (PDT only) in experimental choroidal neovascularization.

Clinical Relevance: This combination therapy can potentially offer a new treatment modality for choroidal neovascularization in patients with macular degeneration and other diseases.

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A GE-RELATED MACULAR DEGENERATION (AMD) is the most common cause of severe, irreversible vision loss in older adults.1 Age-related macular degeneration is a degenerative disease of unknown etiology that affects the macula and can exist in 2 forms: dry (80%-90% of cases) and neovascular or wet (10%-20% of cases).2 Neovascular AMD is characterized by choroidal neovascularization (CNV) in the macular region. Accumulation of serous fluid or blood under the retinal pigment epithelium caused by the increased permeability of newly formed capillaries leads to severe vision loss.3 Discomfort is the fibrotic end stage of the neovascular lesion.4

Although the etiology of AMD remains elusive, there is evidence that vascular endothelial growth factor plays a major role in ocular neovascularization and the pathogenesis of AMD.5-7 Ranibizumab (previously known as rhuFabV2) (Lucentis; Genentech, South San Francisco, Calif) is a humanized anti–vascular endothelial growth factor monoclonal antibody fragment that has been shown to prevent CNV in an experimental monkey model.8 No significant toxic side effects were reported with the injection of ranibizumab in the animal study.9 Ranibizumab is currently under investigation for the treatment of neovascular AMD through intravitreal administration.10 Current therapeutic modalities for AMD are limited to laser photocoagulation and...
photodynamic therapy (PDT). Verteporfin PDT (Visudyne; Novartis, East Hanover, NJ) has been shown to stabilize or slow vision loss in patients with neovascular AMD but requires repeated treatments and may be associated with cumulative damage to normal retinal structures. Combination therapy with PDT and antiangiogenic therapy such as ranibizumab may provide improved vision outcomes for patients.

The present study was designed to evaluate the safety and efficacy of intravitreal injections of ranibizumab combined with verteporfin PDT in laser-induced CNV in the cynomolgus monkey.

## METHODS

### ANIMALS

Cynomolgus monkeys (Macaca fascicularis) were used in accordance with the Association for Research in Vision and Ophthalmology (Rockville, Md) resolution on use of animals in research and in compliance with guidelines developed by the Animal Care Committee of the Massachusetts Eye and Ear Infirmary (Boston). Twelve monkeys were used for this study. Monkeys weighed between 2 and 5 kg, and ages ranged from 1 to 7 years. For the experimental procedures, animals were anesthetized with ketamine hydrochloride (20 mg/kg), acepromazine maleate (0.25 mg/kg), and atropine sulfate (0.125 mg/kg) administered intramuscularly. Supplemental anesthesia was given with ketamine hydrochloride (5–6 mg/kg). Topical ocular anesthesia was obtained with proparacaine. Animals were euthanized with intravenous veterinary pentobarbitol-based euthanasia solution (Vortech Pharmaceuticals, Dearborn, Mich).

### RANIBIZUMAB

Recombinant humanized fragment antigen binding of a monoclonal antibody against human endothelial growth factor (rhuFab vascular endothelial growth factor, rhuFabV2, ranibizumab [Lucentis]) was manufactured by Genentech. It was preserved in a lyophilized powder form in a sterile vial and stored at 2°C to 8°C. The lyophilized powder was reconstituted to either 10 mg/mL or 40 mg/mL in the vial using sterile water for injection and a physiological buffer provided by the manufacturer. We confirmed the final concentration of drug to be injected in the vial and diluted it with 5% dextrose in water, for a total injection volume of 10 mL. Verteoporfin was administered intravenously using a syringe pump over 10 minutes followed by a 2-mL flush of 5% dextrose in water. Fifteen minutes after starting the intravenous infusion of verteporfin, approximately 5 minutes after the end of infusion, the retina was irradiated with a 3000-µm spot size using 689-nm light at 600 mW/cm² and 100 J/cm² using a diode laser from Coherent and laser link apparatus. We treated the second eye within 5 minutes of treating the first eye so that treatments were completed within 20 minutes of the start of verteporfin infusion.

### INDUCTION OF EXPERIMENTAL CNV

Choroidal neovascular membranes were induced in the macula of cynomolgus monkeys with argon green laser burns (Coherent Argon Dye Laser No. 920; Coherent, Santa Clara, Calif) using a Zeiss slitlamp and plano fundus contact lens. Seven lesions were symmetrically placed in the macula of each eye. The laser parameters included 50-µm spot size, 0.1-second duration, and powers ranging from 350 mW to 700 mW. We produced laser-induced CNV in both eyes of 12 cynomolgus monkeys.

### PHOTODYNAMIC THERAPY

Verteporfin for injection (Visudyne; Novartis) was purchased from the manufacturer or an agent of the manufacturer (QLT Inc, Vancouver, British Columbia). The dye was handled, reconstituted, and stored based on the manufacturer’s guidelines. Reconstituted verteporfin was protected from light at all times. We withdrew a dose of 6 mg/m² with a syringe from the vial and diluted it with 5% dextrose in water, for a total injection volume of 10 mL. Verteoporfin was administered intravenously using a syringe pump over 10 minutes followed by a 2-mL flush of 5% dextrose in water. Fifteen minutes after starting the intravenous infusion of verteporfin, approximately 5 minutes after the end of infusion, the retina was irradiated with a 3000-µm spot size using 689-nm light at 600 mW/cm² and 100 J/cm² using a diode laser from Coherent and laser link apparatus. We treated the second eye within 5 minutes of treating the first eye so that treatments were completed within 20 minutes of the start of verteporfin infusion.

### TREATMENT GROUPS

In group 1, 6 animals initially received a 500-µg ranibizumab intravitreal injection in one eye and vehicle (placebo) in the other, starting at day 14 after the laser injury that created CNV (Table 1). This was followed by injecting 2000 µg of ranibizumab in one eye and vehicle (placebo) in the other eye, every 2 weeks for a total of 4 injections. One week after each injection, both eyes underwent verteporfin PDT for a total of 3 treatments. We observed the animals for 2 weeks after the third PDT treatment for a total of 63 days.

In group 2, 4 animals initially received verteporfin PDT in both eyes at day 14 after the laser injury that created CNV, 500 µg of intravitreal injection of ranibizumab in one eye and placebo in the other eye on day 21, and then 2000 µg of ranibizumab every 2 weeks, for a total of 3 injections.
One week after each injection, both eyes underwent verteporfin PDT for a total of 3 treatments. We observed the animals for 2 weeks after the third PDT for a total of 56 days.

Group 3 included 2 animals. Animals received intravitreal injection (ranibizumab in one eye and placebo in the other eye) and verteporfin PDT in both eyes on the same day at 2-week intervals. The treatments started at 2 weeks after the laser injury that induced CNV. The treated eye received 3 injections of ranibizumab and PDT treatments in total. The contralateral eye received placebo intravitreal injection and PDT on the same dosing days. We observed these animals for 2 weeks after the third PDT treatment for a total of 56 days.

SAFETY EVALUATION AND OUTCOMES

Each animal was examined at the slitlamp and with indirect ophthalmoscopy to identify and record inflammation and adverse toxic effects. We did this weekly before performing any treatment (PDT or injection). Anterior chamber and vitreous cells were graded using a 2-mm slitlamp beam at ×16 magnification and using the American Academy of Ophthalmology (San Francisco) scheme. On the day of the ranibizumab dosing, and on the day the monkeys were euthanized, we obtained serum samples from the monkeys. At the time of the enucleation, aqueous and vitreous samples were obtained. Genentech’s bioanalytical assays group analyzed these samples for anti- ranibizumab antibody using a direct enzyme-linked immuno sorbent assay.

OPHTHALMIC EVALUATION AND ANALYSIS

The fluorescein angiograms were graded by 2 masked and experienced examiners (E.S.G. and J.W.M.), who graded by consensus opinion using standard angiograms, as shown in Table 2. This system of grading was developed at the Massachusetts Eye and Ear Infirmary, it was described by Krzystolik et al, and it has been used in various studies in the past. We analyzed the data using a matched-pair analysis with the McNemar χ² test. This evaluated the differences in proportion of eyes with leakage in animals treated with combination therapy (ranibizumab and PDT) in one eye and PDT only in the fellow (control) eye.

HISTOPATHOLOGICAL ANALYSIS

The globes were carefully removed from each animal and dissected clean of orbital tissue. The globes were rinsed in saline and placed in modified Karnovsky fixative consisting of 2% gluteraldehyde and 2.5% formaldehyde in 0.1M cacodylate buffer, 7.4 pH, on ice. Within 10 minutes, we opened the globes and removed the anterior segment. The posterior pole was placed in fixative overnight and then changed to buffer (0.1M cacodylate) until processed for light microscopy.

We prepared each eye for light microscopy by sectioning it into blocks that contained lesions of interest. Tissue was postfixed in 2% osmium tetroxide in 0.1M cacodylate buffer for 2 hours at room temperature and then dehydrated in a series of ethanol, infiltrated with propylene oxide and epoxy resin (Epon), and embed-
We observed less leakage at day 21 after the start of the injection (Table 3). There were no eyes with 3+ or 4+ anterior chamber inflammation or posterior synechiae after the fourth injection. Vitreous cell response as a result of ranibizumab treatment was characterized as a slight increase in vitreous cells (1+ cell) with repeat treatment. The cells were mostly pigmented cells. The combination treatment of ranibizumab and PDT by any regimen either in normal eyes or eyes with laser-induced CNV lesions did not alter this response. Slitlamp biomicroscopy, fundus examinations, fundus photographs, and fluorescein angiography of these eyes did not reveal any other adverse effect of this treatment. The eyes injected with ranibizumab vehicle injection did not show any signs of inflammation.

Animals were divided into 3 treatment groups (1 group received intravitreal ranibizumab first followed by PDT 1 week later, the second group received PDT first followed by intravitreal injection of ranibizumab 1 week later, and the last group received ranibizumab and PDT on the same day). We did this to study whether any order of treatment would have a toxic effect. The number of animals in each treatment group was too small to determine whether one order of treatment was more efficacious than the other. We did not see any adverse event in any group, as detected by slitlamp examination, fundus photography, fluorescein angiography, and histopathology. We studied the safety profile in a total of 12 monkey eyes, which is a modest sample size for nonhuman primate studies. Therefore, the combined treatment appears to be safe in the primate.

### CNV Leakage

We performed a McNemar paired analysis of the data (Table 4) and, for this purpose, combined all the groups. We observed less leakage at day 21 after the start of the first treatment \((P = .25)\) and at day 42 after the start of the first treatment \((P = .50)\) when ranibizumab was injected in combination with verteporfin PDT as compared with PDT used alone. We observed no leakage in animals treated with ranibizumab and PDT at 21 days and 42 days after the first treatment. In contrast, eyes treated with PDT alone showed leakage at 21 days and 42 days after the first treatment in 3 of 12 eyes and 2 of 12 eyes, respectively.

As shown in Figures 1, 2, 3, and 4, at all time points studied, the ranibizumab and PDT–treated eyes experienced better angiographic outcomes than the eyes receiving PDT alone. The CNV leakage was grade 1 or 2 in 100% of combination-therapy eyes at day 14. Also at day 21 after the start of ranibizumab and PDT therapy, 12 (100%) of 12 eyes had grade 1 or 2 leakage at day 35 to the end of the analysis period (day 42). In the PDT-only treatment group, choroidal neovascularization leakage was grade 1 or 2 in 7 (58%) of 12 eyes at day 7, 9 (75%) of 12 eyes at days 14 and 21, and 8 (67%) of 12 eyes at day 28. The number of eyes without leakage increased to 10 (83%) at the day 35 through day 42 follow-up points.

### Histology

Histopathological analysis of eyes with CNV showed that eyes treated with a ranibizumab injection in combination with PDT showed no open blood vessels in the area...
of CNV, whereas PDT-only treated eyes showed rare open blood vessels (Figure 7 and Figure 8). The CNV lesions in both groups consisted of fibrous tissue with macrophages and proliferating retinal pigment epithelium with vacuoles. The architecture of the overlying neurosensory retina depended on how far it was from the original argon laser injury used to create the CNV model. Sections that were at the original laser site showed near complete destruction of the outer retina. Sections away from the original laser scar showed relatively preserved retinal architecture, with shortening of the photoreceptor outer segments and pyknosis of the outer nuclear layer. Therefore, the eyes treated with ranibizumab and PDT showed absence of blood vessels in the CNV, whereas PDT-only eyes showed rare open vessels. Otherwise, there was no significant difference in the size or cellular structure of the CNV in the ranibizumab and PDT treated eyes vs the PDT-only treated eyes.

**COMMENT**

Our data indicate that intravitreal injection of ranibizumab and verteporfin PDT used together appear to be safe and cause greater reduction in angiographic leakage than PDT alone in the laser-injury model of CNV in the monkey. Therefore, combination verteporfin PDT and intravitreal ranibizumab may provide a beneficial effect for patients with neovascular AMD.

We performed intravitreal ranibizumab injections using an initial dose of 500 µg followed every other week by injections of 2000 µg for a total of 3 or 4 injections. This schedule was combined with performing verteporfin PDT every 2 weeks either on the same day as the injection or on alternating weeks with the injection. We observed the animals for 6 to 7 weeks after the first treatment. For the PDT, we used the fluence of 100 J/cm², which was higher than is currently used in clinical practice although it was safely used in earlier clinical trials. Likewise, the application of PDT at 2-week intervals is more frequent than currently used but was also assessed in clinical trials. We chose the higher light dose and short retreatment interval to increase the likelihood of detecting an adverse event and to compare the findings of repeat PDT with those published previously by our group.14

The only potentially adverse reaction noted in our study was transient ocular inflammation in the form of anterior chamber cells. The inflammatory reaction was less severe with subsequent injections and was self-limited. No inflammation was observed in the control eye injected with placebo. These observations are consistent with the safety profiles of ranibizumab as demonstrated in normal.
Figure 5. Fluorescein angiography of experimental model of choroidal neovascularization treated with combination therapy in the right eye and photodynamic therapy (PDT) only in the left eye (group 1 animal). Baseline angiogram shows leakage from the choroidal neovascularization in both eyes. At days 21 and 42 after combination therapy, there is no evidence of leakage from the choroidal neovascularization. In the eye treated with PDT only, there is no leakage at day 21, but late leakage is seen at day 42.

Figure 6. Fluorescein angiography of experimental model of choroidal neovascularization treated with combination therapy in the right eye and photodynamic therapy (PDT) only in the left eye (group 3 animal). Baseline angiogram shows leakage from the choroidal neovascularization in both eyes. At days 21 and 42 after combination therapy, there is no evidence of leakage from the choroidal neovascularization. In the eye treated with PDT only, there is late leakage in the choroidal neovascularization both at day 21 and at day 42.
monkey eyes\(^8\) and monkey eyes with CNV.\(^8\) In the normal monkey eyes, the study drug 2000 µg of ranibizumab was injected intravitreally every other week and followed for 13 weeks. In the study at our laboratory in monkey eyes with CNV,\(^8\) 500 µg of ranibizumab was injected in the eyes every other week, for a total of 4 times.

The ranibizumab injection doses and the frequency of injection in this present study are more aggressive than any previous experimental and clinical doses. Current clinical trials\(^10\) of ranibizumab in patients with macular degeneration have employed lower drug doses. Preliminary results of phase 1 and 2 clinical trials\(^10\) indicate that 300 µg of intravitreal ranibizumab administered every 4 weeks did not show any significant adverse ocular side effects.\(^10\) Similarly repeat PDT treatments have been shown to be safe in monkey studies\(^14\) and also in phase 1 and 2 clinical trials of verteporfin PDT with Visudyne.\(^11\) We carried out this present study using a modest sample size of 12 primate eyes and encountered no adverse event in any eye. Infrequent adverse events may not have been detected and would have required much larger sample sizes, power calculations, and statistical analysis, which was not performed on this smaller group. Nevertheless, we believe that the current study provides sufficient basis to say that combination therapy appears to be safe in primates and that clinical studies may be undertaken. In addition to the data presented here, studies of combination therapy were performed in normal monkey eyes without CNV, and this work will be discussed in a separate article.

In our investigation, the animals were divided into 3 treatment groups. One group received intravitreal ranibizumab first followed a week later by verteporfin PDT, for a total of 4 injections. The second group received verteporfin PDT first followed a week later by intravitreal ranibizumab, for a total of 3 treatments. The third group received verteporfin PDT and intravitreal ranibizumab on the same day (PDT was done first), for a total of 3 treatments. The purpose of using 3 treatment groups was 2-fold. One was to determine whether the order of the treatment showed any adverse event, but we did not see any significant adverse event in any group. Therefore, we believe that any order of treatment is safe. Our second purpose was to provide a guideline for timing the treatments, but the number of animals was too small to definitely recommend 1 group over the other. This question should be studied by clinical trials.

Krzystolik et al\(^{16}\) published study results in the same animal model using ranibizumab, from a study that was divided into 2 phases. In phase 1, intravitreal ranibizumab was injected in 1 eye at day 0, day 14, and day 21, and then both eyes underwent laser to create CNV. Intravitreal ranibizumab was repeated in the treated eye at day 28. Phase 2 of the study began at day 42, when the control eyes were crossed over to the treatment group so both eyes were injected with ranibizumab at day 42 and day 56. This study has shown that intravitreal ranibizumab prevents the formation of CNV with angiographic leakage and that it significantly reduces the leakage in existing CNV. However, grade 4 leakage was still reported in some eyes at day 42 in that study.

In this present investigation, analysis of angiographic leakage from CNV after combination therapy showed that no eyes demonstrated leakage at day 21 and day 42 after the start of treatment. Persistent leakage was observed in 3 (25%) of 12 eyes at day 21 (2 eyes had grade 3 leakage and 1 eye showed grade 4 leakage) and in 2 (16.6%) of 12 eyes (grade 3) at day 42 after PDT alone. Histopathological analysis of the eyes confirmed our angiographic observations in that all eyes in the ranibizumab and PDT combination therapy group showed an absence of vessels in the area of CNV, whereas those that received PDT alone showed rare open capillaries in the lesion.
Although the complete elimination of leakage in the eyes treated with the combination of ranibizumab and PDT suggests that combination therapy may be more effective than intravitreal ranibizumab alone, no direct comparison was made in the current study. Instead, the design of this study compared combination therapy with repeat PDT alone and indicates that there is increased efficacy of combination therapy although we did not achieve statistical significance because of the small sample size. Statistical analysis of this data revealed a larger $P$ value at day 42 than at day 21 (Table 3). This may be due to several factors, including the small sample size (12 animals), the effect of multiple PDT treatments to eliminate leakage, or the inherent tendency for leakage to diminish in this experimental model over time.

In vitro data suggest that prior administration of angiogenic agents potentiates PDT by synergistic cytotoxic damage to endothelial cells. It appears that the administration of ranibizumab in combination with verteporfin PDT may potentiate PDT, affecting CNV regression. This finding remains to be confirmed in clinical studies, and variables such as the optimal timing of ranibizumab administration with respect to PDT have yet to be confirmed. The potential benefit of combination therapy vs ranibizumab alone or PDT alone extends beyond the greater reduction of angiographic leakage. Repeated PDT has shown to result in some irreversible damage to the choriocapillaris and photoreceptor outer segment. Antiangiogenic therapy could reduce the recovery of CNV, which would potentially reduce the number of PDT treatments, leading to less collateral damage and improved outcomes. Similarly, if combination therapy could decrease the frequency and total number of intravitreal ranibizumab injections necessary for CNV treatment, the cumulative risk of complications such as endophthalmitis and hemorrhage could be reduced. Thus, combination verteporfin PDT and intravitreal ranibizumab injection may provide improved therapy for patients with neovascular AMD. Clinical trials of this combination therapy are currently under way according to Joseph C. Beyer, Genentech (written communication, September 2004).

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Financial Disclosure: The Massachusetts Eye and Ear Infirmary is a co-owner of a patent covering the use of verteporfin. In addition, the Massachusetts Eye and Ear Infirmary is the sole owner of claims in patent applications relating to the selective destruction of subretinal choroidal neovascularization for the treatment of macular degeneration and other disorders. Should the Massachusetts Eye and Ear Infirmary receive royalties or other financial remuneration related to the patent and patent filing, Drs Gragoudas and Miller would receive a share of the same in accordance with the Massachusetts Eye and Ear Infirmary's Institutional Patent Policy and Procedures, which includes royalty-sharing provisions. Dr Gragoudas is a consultant for Eyetech Pharmaceuticals (New York, NY). Dr Miller was formerly a consultant for Eyetech Pharmaceuticals and is currently a consultant for Alnylam Pharmaceuticals (Cambridge, Mass), manufacturers of competing products. Dr O'Neill owns stock in Genentech.

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