Comparing Pegaptanib and Triamcinolone Efficacy in the Rat Choroidal Neovascularization Model

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Objective: To evaluate the prophylactic effect of intravitreal pegaptanib sodium on choroidal neovascularization membrane (CNVM) development and compare its performance with that of triamcinolone acetonide.

Methods: In drug-treated and control groups, CNVMs were induced by laser trauma. Immediately after undergoing the laser procedure, animals received intravitreal injections of pegaptanib sodium, 8 or 17 µg; triamcinolone acetonide, 200 µg; or a vehicle solution. After 21 days, fluorescein angiography was performed. The CNVM mean diameters and radial thicknesses were measured histologically.

Results: Mean CNVM diameters were 10% to 13% smaller in pegaptanib-treated eyes and 43% smaller in triamcinolone-treated eyes compared with laser-only control eyes. Late-stage fluorescein angiography leakage scores, on a scale of 0 to 3, suggested a statistical difference between triamcinolone- (0.6) and pegaptanib8 µg-treated (1.5) groups compared with the laser-only control group (2.0). The CNVM mean thicknesses were greater in the pegaptanib8 µg (79 µm) and pegaptanib17 µg-treated (71 µm) groups and significantly smaller in the triamcinolone-treated group (26 µm) compared with the laser-only control group (67 µm).

Conclusion: In this animal model of choroidal neovascularization, intravitreal pegaptanib exhibited marginal or no effect on CNVM development; whereas intravitreal triamcinolone evoked robust inhibition of CNVMs.

Clinical Relevance: Pegaptanib treatment may be insufficient to prevent CNVM formation.

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HOROIDAL NEOVASCULARIZATION (CNV) is an important pathologic component of neovascular age-related macular degeneration. To inhibit CNV development and vascular leakage and perhaps to reverse preexisting neovascularization in patients with neovascular age-related macular degeneration, multiple studies have focused on the development and refinement of angiostatic drug therapy.

Vascular endothelial growth factor (VEGF) is a family within an extended group of cytokine growth factors that under physiologic and pathologic conditions can participate in new blood vessel formation and maintenance.1 Vascular endothelial growth factor–A, consisting of principal amino acid and genetic splice-variant isoforms, has the molecular capability to differentially perform in 1 role or more, depending on the specific tissue, organ, or body location and circumstances, as a multifunctional regulator of neovascularization.2-4 Pegaptanib sodium is an aptamer (RNA oligonucleotide) that demonstrates high-affinity binding with the VEGF-A165 isoform and was the first VEGF antagonist to receive US Food and Drug Administration approval for the clinical treatment of CNV in patients with neovascular age-related macular degeneration.5 To our knowledge, no published reports describe the performance of pegaptanib in an animal model of CNV, although evaluations of pegaptanib have been conducted in other experimental models.6 This study specifically evaluated the prophylactic effect of intravitreal pegaptanib on CNV membrane (CNVM) development and compared its effectiveness with that of the corticosteroid triamcinolone acetonide.

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METHODS

ANIMALS

Male adult (250-g) Brown-Norway rats (Charles River Laboratories, Inc, Wilmington, Massachusetts) were evaluated. All procedures were
performed with strict adherence to guidelines for animal care and experimentation of the Indiana University Animal Care and Use Committee and the Association for Research in Vision and Ophthalmology. For all procedures, including examination and photography, animals received intramuscular ketamine hydrochloride, 50 mg/kg; xylazine hydrochloride, 5 mg/kg; and acepromazine maleate, 1 mg/kg. Topical 1% tropicamide and 2.5% phenylephrine hydrochloride were administered for pupillary dilation and accommodative cycloplegia.

**LASER PHOTOCOAGULATION**

On day 0, animals underwent focal laser photocoagulation placement of sites to induce CNVM formation. After receiving anesthesia and undergoing pupillary dilation and accommodative arrest, the animals were positioned before a slitlamp (Carl Zeiss Meditec, Inc, Jena, Germany) laser delivery system. The fundus was visualized using a microscope slide coverslip with 2.5% hydroxypropyl methylcellulose solution as an optical coupling agent. A diode laser (OcuLight GL; Iris Medical Instrument, Inc, Mountain View, California) was used for photocoagulation (325-nm wavelength; 0.05-second duration; 75-µm spot size; and 120-mW power). In all experiments, a series of 8 photocoagulation sites were concentrically placed around the optic disc of each eye at equal distances (approximately 75-100 µm) from the disc. The placement of small, 75-µm-diameter spots at a moderate laser power of 120 mW most reliably produced acute vapor bubbles, indicative of the rupture of Bruch’s membrane. These experimental conditions also were most favorable for eliciting pronounced CNVMs that often infiltrated the retina.

### DRUG-DOSAGE AND CONTROL GROUP DESIGNATIONS

Drugs evaluated in this study were pegaptanib (Macugen; OSI Eyetech Inc, and Pfizer Inc, New York, New York) and triamcinolone acetonide (in a formulation by QLT Inc, Vancouver, British Columbia, Canada, that excluded preservative agents). Immediately after placement of laser photocoagulation sites, 10 rats in each of 5 designated groups received either an intravitreal injection (5-µL dosage volume) (group 1, pegaptanib sodium17 µg, 16.7 µg of active agent at 100% strength; group 2, pegaptanib sodium16.7 µg, 8.3 µg of active agent diluted to 50% strength; group 3, pegaptanib vehicle (control); and group 4, triamcinolone acetonide, 200 µg) or laser treatment only (LO) (group 5, no-injection control group), to both eyes. Sterile phosphate-buffered saline (BioSource International, Camarillo, California) was used to dilute pegaptanib to a 50% concentration (group 2, pegaptanib16.7 µg, and also served as the pegaptanib vehicle (group 3). Extrapolation from the rat eye (approximately 65 µL of total vitreous and drug volume) containing 8 µg of pegaptanib to that in human eyes (approximately 4 mL of vitreous and drug volume) would be roughly comparable to a 0.5-mg dose of pegaptanib sodium, whereas 17 µg of pegaptanib sodium in the rat eye would approximate a 1.0-mg dose in the human eye. These values fall within the clinically therapeutic dosage range (0.3-3.0 mg) reported for pegaptanib sodium. An additional group of 10 rats received intravitreal injections of the triamcinolone vehicle (group 6) and were compared with the triamcinolone-treated animals and the LO control group.

For intravitreal injections, the sclera was disinfected with 5% povidone iodine. Under stereomicroscopic guidance, a sterile 30-gauge needle was used to penetrate the superior sclera just posterior to the ora serrata. A 32-gauge (Hamilton) needle attached to a microsyringe was advanced through the scleral opening into the vitreous cavity.

**ASSESSMENT**

All animals underwent fundus photography and fluorescein angiography (model FK 30 fundus camera; Carl Zeiss Meditec, Inc) just before euthanasia on day 21. For fluorescein angiographic evaluations, 10% sodium fluorescein, 10 mg/kg, was administered intravenously and photographs were taken at predesignated times (1-10, 15, 30, 60, 90, 180, and 300 seconds). Scoring of late-phase (90 or 180 seconds) vascular staining and leakage was based on a whole-number fluorescein angiography grading scale that ranged from 0 (none or slight) to 3 (severe). Mean scores for each drug and control group represented the mean of the total cumulative scores at the 4 grades divided by the number of identified lesion sites. The angiograms were subjected to masked analysis for the presence and intensity of staining and for leakage at each lesion site.

To determine lateral CNVM spread from these images, the diameter of each site was measured along the projected axis, which extended from the lesion center to the optic disc (Figure 1). Measurements along this axis were optimal for overall continuity in data collection, for group data comparisons, and to reduce possible errors caused by CNVM spread between adjacent sites.

**Figure 1.** To measure lateral choroidal neovascular membrane (CNVM) spread, the diameter of each site (arrow) was measured along the projected axis (dotted line) that extended from the lesion center to the optic disc. Measurements along this axis were optimal for overall continuity in data collection, for group data comparisons, and to reduce possible errors caused by CNVM spread between adjacent sites.
crete lesion sites that resulted from lateral CNVM expansion from one lesion site into the next.

Histologic tissues from treatment and vehicle control groups were methodically assessed in a masked fashion to analyze each laser lesion for the presence or absence of experimental CNVs. Each recovered lesion site was evaluated in its entirety to quantitate the extent of fibrovascular proliferation. Maximum CNVM thickness was measured from the choriocapillaris, which at some control sites had expanded distally into the deeper choroidal or inner scleral layers. Proximally, CNVM development remained subretinal at some lesion sites, whereas at other sites, encroachment continued proximally with partial infiltration of the retina. Occasionally, individual neovessels from the central CNVM mass continued to infiltrate further into the retinal layers; however, this vasculature was not included in the CNVM thickness measurements. Identification of CNV within the membrane was verified qualitatively by vessel diameter, path through sequential serial tissue sections, and the presence of red blood cells in the lumen. Further confirmation of new vessel formation within the CNVM previously was determined by histologic-labeling techniques: CD31 antigen, fluorescein isothiocyanate–albumin, and rhodamine–cationic liposomes.9

For statistical analyses, no zero CNVM thickness values were permitted. The thickness of the adjacent choriocapillaris layer provided a minimum CNVM site thickness value. The CNVM thickness measurements for each group were obtained using the maximum thickness measurements of each of the approximately 8 recovered CNVM lesion sites per eye. Drug treatment and control vehicle group means (SDs) were determined. One-way analysis of variance and Tukey-Kramer honestly significant difference tests were used to compare the significance of mean differences and variance between the groups. P < .05 was considered statistically significant.

Table 1. Comparison of CNVM Mean Diameter

<table>
<thead>
<tr>
<th>Group</th>
<th>Diameter, mean (SD), µm</th>
<th>Reduction, %(^a)</th>
<th>P Value(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triamcinolone acetonide</td>
<td>112 (36)</td>
<td>43</td>
<td>&lt;.01: All other groups</td>
</tr>
<tr>
<td>Pegaptanib sodium, 8 µg</td>
<td>170 (43)</td>
<td>13</td>
<td>&lt;.05: Pegaptanib vehicle;</td>
</tr>
<tr>
<td>Pegaptanib sodium, 17 µg</td>
<td>176 (43)</td>
<td>10</td>
<td>&lt;.05: LO;</td>
</tr>
<tr>
<td>Triamcinolone vehicle</td>
<td>189 (41)</td>
<td>4</td>
<td>&lt;.01: Triamcinolone</td>
</tr>
<tr>
<td>Pegaptanib vehicle</td>
<td>192 (39)</td>
<td>2</td>
<td>&lt;.01: Triamcinolone</td>
</tr>
<tr>
<td>LO</td>
<td>196 (39)</td>
<td>. . .</td>
<td>&lt;.01: Triamcinolone</td>
</tr>
</tbody>
</table>

Abbreviations: CNVM, choroidal neovascularization membrane; LO, laser treatment only; ellipses, not applicable.

\(^a\) Compared with LO control group mean.

\(^b\) One-way analysis of variance and Tukey-Kramer test.

The CNVM mean diameter was not significantly different in the pegaptanib\(_{17 \text{ µg}}\)-treated group (176 µm) compared with the pegaptanib vehicle group (192 µm) but was significantly different (10% reduction; \(P < .05\)) compared with the LO group (196 µm). There was a small (14%) but statistically significant (\(P < .01\)) reduction in mean CNVM diameter in the pegaptanib\(_{8 \text{ µg}}\)-treated group (170 µg) compared with the LO group, whereas the triamcinolone group (112 µm) demonstrated a 43% reduction in CNVM diameter compared with the LO group and significant differences (\(P < .01\)) compared with all other groups. (Table 1).

Qualitative visual examination of late-phase vascular staining and leakage revealed no overt differences in fluorescein leakage between the pegaptanib-treated and control groups (Figure 2A-E). Approximately 70% of triamcinolone-treated eyes contained sites that expressed localized fluorescein that resulted from leakage limited to the area immediately in and around the photocoagulation sites. In the remaining triamcinolone-
treated eyes, late-phase fluorescence at photocoagulation sites had become faint and dye staining had dissipated (Figure 2F). Analyses of the fluorescein angiography–graded scores revealed no significant differences in mean scores for staining between the pegaptanib17 µg-treated (mean score, 1.7) and the pegaptanib vehicle (1.7) or LO (2.0) groups (Table 2). The pegaptanib8 µg-treated group score (1.5) also was not significantly different from either pegaptanib vehicle or triamcinolone vehicle groups but was significantly (P < .01) lower compared with the LO group. The triamcinolone score (0.6) was significantly lower compared with all other groups. The percentage of sites in the pegaptanib-treated and control groups that exhibited substantial leakage (grades 2 and 3) ranged from 54% in the pegaptanib8 µg-treated group to 79% in the LO group compared with only 4% in the triamcinolone-treated group (Table 2).

Histopathologic examination of radial tissue sections did not reveal any discernible qualitative differences between the control and pegaptanib-treated groups (Figure 3). Patent neovessels were evident and common in the CNVMs of eyes treated with pegaptanib17 µg and pegaptanib8 µg, often with infiltration that continued into the retina (Figure 3D and E and Figure 4A). Anastomotic vessel development between the choroidal and retinal vascular layers also was common in CNVMs and in neighboring retinal tissues in both the control and pegaptanib groups. In more than 97% of the triamcinolone-
lone-treated sites, CNVMs did not develop within the central laser-trauma site. Rather than development of a CNVM, the retina is drawn distally into the defect. Immediately adjacent to the lasered site, some thickening of the choriocapillaris layer was observed that may have resulted from localized edema. Quantitatively, CNVM and choriocapillaris mean thicknesses (Table 3) were greater in the pegaptanib 8 µg- (79 µm) and pegaptanib 17 µg-treated (71 µm) groups and were significantly less in the triamcinolone-treated group (26 µm).

To assess the direct antiangiogenic response of triamcinolone from a possible effect of the intravitreal injection, triamcinolone was compared with its vehicle. Whereas triamcinolone elicited a reduction of approximately 43% in mean CNVM diameter compared with the LO control group, the triamcinolone vehicle group demonstrated only a 4% difference compared with the LO control group (P < .01). Similarly, although triamcinolone administration resulted in a reduction of approximately 61% in CNVM mean thickness compared with the LO control group, the difference between the triamcinolone vehicle group and the LO control group was only 6% (P < .01).

Table 3. Comparison of CNVM Mean Radial Thickness

<table>
<thead>
<tr>
<th>Group</th>
<th>Radial Thickness, Mean (SD), µm</th>
<th>Reduction, % a</th>
<th>P Value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triamcinolone acetonide</td>
<td>26 (10)</td>
<td>61</td>
<td>&lt;.01: All other groups</td>
</tr>
<tr>
<td>LO</td>
<td>67 (20)</td>
<td>. . .</td>
<td>&lt;.01: Pegaptanib sodium, 8 µg, and triamcinolone</td>
</tr>
<tr>
<td>Pegaptanib sodium, 17 µg</td>
<td>71 (17)</td>
<td>(6)</td>
<td>&lt;.01: Pegaptanib sodium, 8 µg, and triamcinolone</td>
</tr>
<tr>
<td>Triamcinolone acetonide vehicle</td>
<td>71 (20)</td>
<td>(6)</td>
<td>&lt;.05: Pegaptanib sodium, 8 µg;</td>
</tr>
<tr>
<td>Pegaptanib vehicle</td>
<td>72 (14)</td>
<td>(7)</td>
<td>&lt;.01: Triamcinolone</td>
</tr>
<tr>
<td>Pegaptanib sodium, 8 µg</td>
<td>79 (19)</td>
<td>(18)</td>
<td>&lt;.01: LO, Pegaptanib sodium, 17 µg, and triamcinolone acetonide</td>
</tr>
</tbody>
</table>

Abbreviations: CNVM, choroidal neovascularization membrane; LO, laser treatment only; ellipses, not applicable.
a Compared with LO control group mean. Values in parentheses denote negative values (ie, values greater than LO values).
b One-way analysis of variance and Tukey-Kramer test.

Vascular endothelial growth factor–A164 is the murine homologue of the VEGF-A165 human isoform.2 Pegaptanib recognizes and reacts to VEGF-A164/165.5,6 Inhibition of CNV should have been a predictable outcome of pegaptanib administration in the rat model, but this did not occur. In a primate laser-trauma CNV model, intravitreal pegaptanib also did not inhibit neovascularization.10 Why did pegaptanib fail to block CNV development? Numerous factors may have contributed to this result. Neovascularization is not a uniform process throughout the body, and the regulatory functions of VEGF may vary depending on where neovascularization is occurring and under what circumstances.2,11,12 In CNV, the specific role(s) and extent of involvement of VEGF have not been defined with confidence. Regulatory differences also may exist between various types of CNV, ocular neovascularization (eg, corneal and iridial), and retinal neovascularization.13 Experimental findings can be affected by the animal models and species used.13 Animal and human re-
VEGF-A can evoke an antiangiogenic effect on CNV even before choroidal injury, there is a period during which there is a quiescent period of angiogenesis. Recent evidence also suggests that the remaining VEGF-A isoforms may continue to mediate angiogenesis. Pegaptanib blocks both isoform variants, it is conceivable that vascular endothelial growth factor–A isoforms exhibit differences in their biochemical and receptor binding properties. Pegaptanib binds with the secreted forms of VEGF-A165, rendering it unable to activate receptor sites. Alternatively, these receptors still can be activated by VEGF-A120 (murine and human) found in the normal adult retina. Although VEGF-A120 exhibits a mitotic potency 10 to 100 times less than that of VEGF-A165, there is only a 2-fold difference in its potency as a mediator of retinal leukostasis and blood-retinal barrier breakdown.

During its original development, pegaptanib was found to inhibit VEGF-A165 but not VEGF-A121 proliferation. Pegaptanib is truncated and modified to render it more effective in decreasing VEGF-induced vascular permeability, consequently reducing its VEGF binding affinity approximately 4-fold. In clinical trials, pegaptanib reportedly exerted “a slowing” of total lesion and CNV size, as well as of “the severity of leakage.” While it reportedly demonstrated an effect in preventing moderate visual acuity loss.

Larger VEGF isoforms that are bound by heparin to the cell membrane and extracellular matrix, such as VEGFA144, can be secreted from source cells and also can occur in 2 bound states to the cell membrane and extracellular matrix. Pegaptanib binds with the secreted forms of VEGF-A165, rendering it unable to activate receptor sites. Alternatively, these receptors still can be activated by VEGF-A120 (murine and human) found in the normal adult retina. Although VEGF-A120 exhibits a mitotic potency 10 to 100 times less than that of VEGF-A165, there is only a 2-fold difference in its potency as a mediator of retinal leukostasis and blood-retinal barrier breakdown.

Vascular endothelial growth factor–A isoforms exhibit differences in their biochemical and receptor binding properties. Vascular endothelial growth factor–A isoforms exhibit differences in their biochemical and receptor binding properties. In addition to VEGF-A165, pegaptanib potentially could bind with VEGF-A165, a human splice-variant isoform that inhibits VEGF-mediated angiogenesis. If pegaptanib blocks both isoform variants, it is conceivable that proangiogenic and antiangiogenic actions of these molecules could be suppressed.

Another possibility is that VEGF may not be an effective or critical mitogen at the onset of CNV. Other multifunctional regulators that can function both in parallel and synergistically with VEGF are upregulated. Whether the selective elimination of the VEGF-A165 isoform is sufficient to prevent CNV development is uncertain.

Hypothetically, the role of VEGF at the onset of CNV formation may be less critical than that in retinal neovascularization in diabetic retinopathy because high blood glucose levels and retinal ischemia elevate VEGF levels. pegaptanib may affect vascular permeability and maintenance, but this requires further evaluation. The precise relationships of the various VEGF isoforms to CNV development, vascular stability, and hyperpermeability need to be clearly defined to optimize therapeutic intervention while not adversely affecting normal and necessary physiologic functions in the eye and systematically.

The findings of this investigation indicate that pegaptanib as a selective inhibitor of VEGF-A165 is insufficient to singularly prevent new CNVM formation (Figure 4). Given the small but statistically significant effect of pegaptanib on CNVM diameter and vascular leakage in this investigation, pegaptanib may exert a partial influence on the motogenic spread of neovascularization or some other effect on vascular hyperpermeability. Whether pegaptanib may affect the maintenance and survival of preexisting CNV should be explored.

Although triamcinolone effectively inhibited CNV in this model and reportedly decreased membrane diameter in a clinical study, its clinical effect on vision remains “controversial.” Optimal dosing levels and frequency of administration have not been well established clinically, and corticosteroid therapy is associated with its own set of potential short- and long-term risks.

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Additional Contributions: Lisa Bird-Turner, AS, provided technical expertise and assistance with the histopathologic analysis.
REFERENCES


