The Effects of Intravitreous Bevacizumab on Retinal Neovascular Membrane and Normal Capillaries in Rabbits

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PURPOSE. To evaluate the effects of intravitreous bevacizumab in a rabbit retinal neovascularization model.

METHODS. Twenty-four rabbits were divided into five groups. Group A included four rabbits; all other groups included five rabbits each. Group A received intravitreous VEGF only, and group E received intravitreous bevacizumab only. In groups B, C, and D, bevacizumab was injected at the same time, at day 2 and at week 1 after VEGF injection, respectively. Follow-up evaluations continued for 3 weeks and included color fundus photography, fluorescein angiography (FA), and optical coherence tomography (OCT). Enucleated eyes were processed for hematoxylin and eosin (H&E) staining.

RESULTS. Intravitreous VEGF was associated with disc hyperemia, vascular dilatation and tortuosity, and fluorescein leakage at the disc and in the anterior chamber (AC) at day 2 and with formation of retinal neovascular membranes (NVM) by week 1. At weeks 2 and 3, the NVM was replaced by a fibrotic membrane and mild to moderate capillary nonperfusion. In groups B and C, injection of bevacizumab was very effective in preventing or stopping fluorescein leakage but was not able to prevent or reverse vascular dilatation and tortuosity completely. In group D, bevacizumab injection resulted in severe capillary nonperfusion at week 2.

CONCLUSIONS. Intravitreous injection of VEGF in rabbits results in florid retinal neovascularization within the first week, followed by closure of normal capillaries by week 2. Early intravitreous injection of bevacizumab can prevent these effects, whereas late injection may be associated with more significant closure of normal capillaries. A sudden drop in effective VEGF concentration may be responsible for the closure of the normal capillaries. (Invest Ophthalmol Vis Sci. 2007;48:5708–5715) DOI:10.1167/iovs.07-0731

Vascular endothelial growth factor (VEGF), a 35- to 45-kDa homodimeric protein initially known as vascular permeability factor, is a potent stimulator of endothelial cell growth and neovascularization and has a strong permeability effect on vessels.1-4 The VEGF gene family consists of VEGF-A, -B, -C, and -D and placental growth factor (PlGF), which have different binding affinities for the three VEGF receptors (VEGFR).5 VEGF, as it is classically known, refers to VEGF-A, which binds to VEGFR-1 and -2, which are primarily involved in angiogenesis.6-8 VEGF-A in turn has several isoforms of which VEGF165 is one of the most abundant.5 Increased VEGF levels in the vitreous of eyes with diabetic retinopathy, retinal vascular occlusions, and subretinal neovascularization have implicated VEGF as the major stimulus for intraocular neovascularization in these conditions.9,10

Bevacizumab is a humanized murine anti-VEGF monoclonal antibody that is specific to human VEGF and blocks all its isoforms, including VEGF165.5,11 Bevacizumab was developed for cancer treatment and has demonstrated improved survival in the treatment of metastatic colorectal cancer, advanced non-small-cell lung cancer and metastatic breast cancer.12,13 Recently, intravitreous bevacizumab has been used extensively for the treatment of macular edema and neovascularization in diseases such as diabetic retinopathy, age-related macular degeneration, retinal vein occlusion, neovascular glaucoma, and many other conditions.14-24 Several safety studies have reported the lack of toxicity of intravitreous bevacizumab in rabbits.25-28 However, more recent studies have demonstrated ultrastructural changes after intravitreous injection of bevacizumab in rabbits and primates.29,30 These previous toxicity studies have all injected bevacizumab into normal eyes. The purpose of our study was to evaluate the effects of intravitreal bevacizumab in an animal neovascularization model.

MATERIALS AND METHODS

Animals

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of Southern California.

Twenty-four pigmented rabbits, weighing 2 to 3 kg, were used in the study. Only the right eye of each animal was used. Rabbits were assigned to five groups: groups A to D received intravitreal VEGF (recombinant human VEGF165; Sigma-Aldrich, St. Louis, MO). Groups B, C, and D (n = 5 each) received intravitreal bevacizumab (Avastin; Genentech Inc., San Francisco, CA) immediately after the VEGF injection, 2 days later, and 1 week later, respectively. Group A (n = 4) did not receive any further injection and was used as the control. Group E (n = 5), another control arm, received intravitreal bevacizumab only. The follow-up period was 3 weeks.

Intravitreal Injections

First, the rabbits were anesthetized with a subcutaneous injection of a mixture of ketamine hydrochloride (25 mg/kg) and xylazine hydrochloride (6 mg/kg). The pupils were dilated with topical application of phenylephrine hydrochloride 2.5% and tropicamide 0.5% eye drops.

Intravitreal injections were performed in sterile conditions and after instillation of several drops of 5% povidone iodine. Topical teta-
caine drops were applied for additional anesthesia. A speculum was used to keep the eyelids open, and the procedure was performed under direct visualization using an operating microscope and a surgical contact lens. A 27-gauge needle attached to a 1-mL syringe was introduced into the vitreous cavity transconjunctivally, at the superotemporal quadrant, 1.5 mm behind the limbus. If the total volume of the intravitreous drugs to be injected was to be more than 0.05 mL (i.e., as in group B), the same volume of vitreous was first withdrawn to prevent extravasation of the drug from the injection site. Intravitreous injection was then performed from the same port, using another 27-gauge needle and a 1-mL syringe. In group B, which received VEGF and bevacizumab at the same time, bevacizumab was injected from a different port, at the superonasal quadrant, 1.5 mm behind the limbus. The amount of VEGF injected was 10 μg in 0.01 mL and that of bevacizumab was 1.25 mg in 0.05 mL. At the end of the procedure, topical antibiotic ointment (Neo-Poly-Bac; Bausch & Lomb Inc., Tampa, FL) was applied.

Follow-up Examinations

Examinations included evaluation of the anterior segment by surgical microscope, color fundus photography, fluorescein angiography (FA), and optical coherence tomography (OCT). Before each session, the rabbits were anesthetized, and the pupils were dilated as explained earlier. Baseline examinations were performed within a week before the first intravitreous injection, and follow-up examinations were performed at day 2 and weeks 1, 2, and 3 after the first injection.

Color Fundus Photography. A digital fundus camera system (model FF 450 IR; Carl Zeiss, Jena, Germany) was used to take at least five photographs in each eye: optic disc, temporal medullary wing, nasal medullary wing, superior retina above the disc, and inferior retina below the disc.

Fluorescein Angiography. The same digital fundus camera system used for color fundus photography was set for FA by selecting the appropriate filters. An intravenous line was established on the marginal ear vein and 0.2 mL of 10% fluorescein was injected and continued until the last frames at 5 minutes. At week 1, the leakage persisted and, in addition, a well-developed neovascular membrane was visible on color fundus photography and FA (Figs. 1, 3). Anterior segment examination or FA failed to show clear iris neovascularization, despite evidence of leaking on FA. OCT showed epiretinal thickening over the medullary wings (Fig. 2).

Histopathology

At the end of the last follow-up examination in each group, the rabbits were euthanatized by intracardiac injection of 2 mL pentobarbital (Beuthanasia-D; Schering Plough Animal Health, Omaha, NE). The eyes were enucleated and immersed in Davidson’s fixative solution for 16 to 24 hours and then dehydrated in a series of graded alcohol solutions in the next 24 to 48 hours before paraffin embedding. Blocks were obtained from cuts through the whole globe, oriented perpendicular to the medullary wings. Sections 5 μm thick obtained by a microtome were stained with hematoxylin and eosin (H&E) and examined by light microscopy.

RESULTS

Intravitreous injection of VEGF was invariably associated with disc hyperemia, vascular dilatation and tortuosity, and fluorescein leakage at the optic disc, medullary wings and anterior chamber (AC) at day 2 (Table 1, Figs. 1, 2, 3). The leakage started early, approximately 20 seconds after intravenous fluorescein injection, and continued until the last frames at 5 minutes. At week 1, the leakage persisted and, in addition, a well-developed neovascular membrane was visible on color fundus photography and FA (Figs. 1, 3). Anterior segment examination or FA failed to show clear iris neovascularization, despite evidence of leaking on FA. OCT showed epiretinal thickening over the medullary wings (Fig. 2).

| Table 1. Color Fundus Photography and FA Findings in Groups A, B, C, and D |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Bevacizumab Injections | Fundus Photo | FA | Fundus Photo | FA | Fundus Photo | FA | Fundus Photo | FA | Fundus Photo | FA | Fundus Photo | FA |
| None | A1 | DH, VDT | L | DH | L, NVM | DP | RDE, VDT, AVP | Moderate CN | DP | RDE, VDT, AVP | Moderate CN |
| A2 | DH, VDT | L | DH | L, NVM | DP | RDE, AVP | Moderate CN | DP | RDE, AVP | Moderate CN |
| A3 | DH, VDT | L | DH | L, NVM | DP | RDE, VN, AVP | Mild CN | DP | RDE, VN, AVP | Mild CN |
| A4 | DH, VDT | L | DH | L, NVM | DP | RDE, VN, AVP | Mild CN | DP | RDE, VN, AVP | Mild CN |
| Day 0 | B1 | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT |
| B2 | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT |
| B3 | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT |
| B4 | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT |
| B5 | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT | DH, VDT |
| Day 2 | C1 | DH, VDT | L | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT |
| C2 | DH, VDT | L | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT |
| C3 | DH, VDT | L | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT |
| C4 | DH, VDT | L | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT |
| C5 | DH, VDT | L | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT | VDT |
| Week 1 | D1 | DH, VDT | L | DH | L, NVM | DP | RDE, VN, AVP | Severe CN | DP | RDE, VN, AVP | Severe CN |
| D2 | DH, VDT | L | DH | L, NVM | DP | RDE, VN, AVP | Severe CN | DP | RDE, VN, AVP | Severe CN |
| D3 | DH, VDT | L | DH | L, NVM | DP | RDE, VN, AVP | Severe CN | DP | RDE, VN, AVP | Severe CN |
| D4 | DH, VDT | L | DH | L, NVM | DP | RDE, VN, AVP | Severe CN | DP | RDE, VN, AVP | Severe CN |
| D5 | DH, VDT | L | DH | L, NVM | DP | RDE, VN, AVP | Moderate CN | DP | RDE, VN, AVP | Moderate CN |

DH, disc hyperemia; VDT, vascular dilatation and tortuosity; L, leakage at disc, medullary wings and AC; NVM, neovascular membrane; VN, vascular narrowing; DP, disc pallor; AVP, abnormal vascular pattern; RDE, retinal distortion and elevation; CN, capillary nonperfusion.
In group A, which was kept as control, there was no leakage at the optic disc or in the AC at week 2 (Fig. 3). In addition, no neovascular membrane was visible on color fundus photography or FA (Fig. 1). OCT, however, showed thinning of the epiretinal membrane (Fig. 2). At this time, the optic disc and medullary wings were pale, the retinal surface at medullary wings near the optic disc was irregularly elevated, the vascular pattern was irregular, and there was vascular narrowing in some cases. At week 3, there was no significant change, except that horizontal retinal folds appeared at the upper and lower edges of the medullary wings. There was mild to moderate capillary nonperfusion in all cases (Table 1; Figs. 1, 3). Vascular dilatation and tortuosity diminished slightly at week 3 but was not completely resolved.

In group B, in which bevacizumab was injected at the same time as VEGF, no leakage occurred at the optic disc or in the AC at day 2 or beyond, but the retinal vessels became mildly dilated, and tortuous and retinal capillaries became prominent (Table 1; Figs. 1, 3). Vascular dilatation and tortuosity diminished slightly at week 3 but was not completely resolved.

In group C, in which bevacizumab was injected 2 days after VEGF, there was no leakage at the optic disc or in the AC at week 1 or beyond, nor was there formation of any neovascular membrane. There was, however, mild vascular dilatation and tortuosity, and prominence of the retinal capillaries (Table 1; Figs. 1, 3). Vascular dilatation and tortuosity diminished slightly at week 3 but was not completely resolved.

In group D, in which bevacizumab was injected 1 week after VEGF, color fundus photography and FA findings were similar to those in group A at week 2, except that the capillary nonperfusion was severe in four of the five animals in this group (Table 1, Figs. 1, 3, 4).

Group E, which received intravitreous bevacizumab only, did not show any abnormalities in color fundus photography or FA over time (Figs. 1, 3).

In groups A and D there was epiretinal fibrosis over the medullary wings in all cases (Fig. 5). The membrane was associated with retinal folds at the upper and lower edges of the medullary wings. Groups B and C showed vascular crowding over the medullary wings. Group E did not display any abnormality.

**DISCUSSION**

Several investigators have created retinal neovascularization in rabbits using sustained-release pellets loaded with VEGF with or without basic fibroblast growth factor (bFGF). Others have used bevacizumab to block VEGF. In our study, however, we used a simpler retinal neovascularization...
model that we developed. In this model, a single intravitreous VEGF injection is used to generate retinal neovascularization within a week. The VEGF dose (10 μg) was empirically chosen, as it was found to be consistent with a study that showed that 10.70 ± 0.92 μg/mL of sustained-release VEGF causes retinal neovascularization between 12 and 18 days after implantation of sustained-release devices. Further release of VEGF in that study resulted in retinal detachment.32

**FIGURE 4.** Representative FA images in groups A to E showing capillary nonperfusion at week 2. Note patches of capillary nonperfusion in group A (arrows), vascular and capillary dilatation and tortuosity in groups B and C, and diffuse nonperfusion in group D.

**FIGURE 5.** H&E staining of cross sections of the medullary wings near the optic disc, comparing a normal rabbit group N with representative rabbits in groups A to E. Note the epiretinal fibrosis and retinal folds at the upper and lower edges of the medullary wings in groups A and D, and vascular crowding in groups B and C (separation of vessels from the retinal surface in group C is an artifact). Magnification, ×10.
When bevacizumab was injected at the same time as VEGF or 2 days later, retinal neovascularization was prevented. In addition, bevacizumab treatment was able to prevent or stop leakage both at the disc and in the AC. However, it was not able to prevent or reverse vascular dilatation and tortuosity completely. Some investigators have speculated that the amount and duration of VEGF exposure necessary for blood-retina barrier breakdown may be less than that for neovascularization. Our study did not support or reject this idea. However, assuming that vascular dilatation and tortuosity could be a part of early stage of neovascularization, it may be that the amount of VEGF required for initiation of neovascularization is less than that for blood–retina barrier breakdown. Little is known about the role of VEGF in the maintenance of adult ocular vasculature. Although some studies have indicted that VEGF maintains normal vasculature in the respiratory system of mice and rats, a study of adult mice did not find the normal retinal vasculature to be dependent on VEGF. On the other hand, it has been shown that VEGF withdrawal can lead to the regression of the newly grown vessels but not the more established ones. In our study, in the positive control group (group A), intravitreous injection of VEGF resulted in formation of a neovascular membrane at week 1. However, at week 2, the membrane became avascular, and in addition, there was mild to moderate nonperfusion of normal capillaries. Injection of bevacizumab 1 week after VEGF injection (group D) was associated with more extensive nonperfusion and closure of normal capillaries at week 2. It seems that after week 1, there was a reverse correlation between the effective VEGF concentration and the extent of capillary nonperfusion. In other words, blockage of the remaining VEGF by bevacizumab resulted in a sudden decrease in effective VEGF concentration and consequently a more extensive capillary nonperfusion.

Repeated intravitreous injection of VEGF in primates has been shown to be associated with retinal capillary nonperfusion secondary to endothelial cell hyperplasia and hypertrophy. However, no capillary nonperfusion has been observed after a single intravitreous injection of VEGF. In our study, capillary nonperfusion occurred between the first and second weeks after a single intravitreous VEGF injection, when the VEGF concentration was falling. This observation suggests that normal vessels may become VEGF dependent when suddenly exposed to a high concentration of VEGF. It seems a certain amount of VEGF is necessary to maintain the newly grown vessels as well as normal, but now sensitized, capillaries. When the effective VEGF concentration declines below this putative threshold, it may result in regression of the new vessels and closure of normal capillaries.

The mechanism by which normal capillaries become VEGF dependent is not clear. Although downregulation of VEGF receptors in endothelial cells may play a role, it is plausible that the strong VEGF drive causes sprouting of numerous new vessels from the normal capillaries such that normal capillaries transform and become part of the architecture of the neovascular membrane and hence VEGF dependent. This possibility is supported by the FA at week 1 which illustrates that the majority of normal capillaries are indistinguishable from the newly formed vessels within the neovascular membrane (Fig. 3).

Vascular response to anti-VEGF therapy appears to be related to the degree of vessel maturity (e.g., presence of pericytes and vascular smooth muscle cells around capillaries and larger vessels, respectively). Mural cell recruitment to endothelial cells requires a signaling process involving platelet-derived growth factor (PDGF)-B, produced by endothelial cells, and PDGF receptor (PDGFR)-, carried by mural cells or un-differentiated mesenchymal cells. Tumor vessels lacking mu-

ral cells are more sensitive to VEGF-A withdrawal-induced apoptosis. Thus, mature vessels may be less sensitive to anti-VEGF-A therapy. In animal models of choroidal neovascularization (CNV), combined inhibition of VEGF-A and PDGF-B signaling pathways via pegaptanib and an antibody against PDGFR-, respectively, synergistically inhibits CNV growth.

Although further studies are necessary to elucidate the mechanisms of the observed phenomenon, the significant nonperfusion observed in this study would raise concerns regarding the use of anti-VEGF therapy in states in which high levels of VEGF are present (e.g., in diseases with severe retinal ischemia). Thus, until additional data are available, judicious use of anti-VEGF drugs such as bevacizumab and ranibizumab may be warranted in cases of acute and severe retinal neovascularization, to prevent capillary nonperfusion and further retinal ischemia. The significance of the findings of the present study, however, is limited by the nature of the animal model (the merangiotic rabbit retina), in which the retinal vasculature is on the surface of the retina and not within the retina as in humans. The relevance to human retinal vascular disease remains uncertain until these results can be confirmed in other model systems. Another limitation of our study is that the experimental pathology is solely induced by VEGF which is believed to be the predominant factor but not the only VEGF isoform involved in neovascular diseases. Indeed, VEGF, although less abundant, is more mitogenic than VEGF. In addition, all other VEGF isoforms theoretically may be cleaved by plasmin to generate VEGF which can stimulate endothelial cell growth and induce vascular permeability.

From a different perspective, the observed phenomenon in our study could be used to sensitize established new vessels to antiangiogenesis drugs by exposing them to a high dose of VEGF. This method of sensitization may be of use in treating established neovascular membranes and ocular or systemic tumors.

Histopathologic examination of the enucleated eyes showed epiretinal fibrosis after disappearance of neovascular membranes in groups A and D. This finding is consistent with the clinical outcome observed in patients with proliferative diabetic retinopathy treated by laser photocoagulation or intravitreous bevacizumab and with a histopathological report of a human case of choroidal neovascular membrane treated by intravitreous bevacizumab.

In conclusion, intravitreous injection of VEGF resulted in formation of a neovascular membrane at week 1, followed by regression of the new vessels and mild to moderate closure of normal capillaries at week 2. Early intravitreous injection of bevacizumab (at the same time as VEGF injection or two days later) was able to prevent or stop leakage both at the disc and in the AC but was not able to prevent or reverse vascular dilatation and tortuosity completely, indicating that a lower dose of VEGF may be necessary for initiation of neovascularization than for blood–retina barrier breakdown. Of importance, late injection of bevacizumab (1 week after VEGF injection) resulted in severe closure of normal capillaries. This effect may be due to normal capillaries becoming VEGF dependent when suddenly exposed to a high dose of VEGF. Thus, in treating patients with acute or severe retinal neovascularization with anti-VEGF drugs such as bevacizumab, caution may be warranted to prevent capillary nonperfusion and macular ischemia. Further studies are needed to evaluate the possibility of sensitizing established neovascular membranes or established new vessels in ocular or systemic tumors to antiangiogenesis drugs by exposing them to a high dose of growth factors such as VEGF.
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