ELECTROPHYSIOLOGIC AND RETINAL PENETRATION STUDIES FOLLOWING INTRAVITREAL INJECTION OF BEVACIZUMAB (AVASTIN)

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Purpose: Intravitreal bevacizumab (Avastin; Genentech Inc., San Francisco, CA) is a new treatment for age-related macular degeneration. The aim of this study was to evaluate retinal penetration and toxicity of bevacizumab.

Methods: Ten albino rabbits were injected intravitreally with 0.1 mL (2.5 mg) of Avastin to one eye and 0.1 mL saline into the fellow eye. The electroretinogram (ERG) was recorded after 3 hours, 3 days, and 1, 2, and 4 weeks. The visual evoked potential (VEP) was recorded after 4 weeks. Confocal immunohistochemistry was used to assess retinal penetration.

Results: The ERG responses of the control and experimental eyes were similar in amplitude and pattern throughout the follow-up period. The flash VEP responses of the experimental eyes were of normal pattern and amplitude and did not differ from those recorded by stimulation of the control eye alone. Full thickness retinal penetration was present at 24 hours and was essentially absent at 4 weeks.

Conclusions: Bevacizumab was found to be nontoxic to the retina of rabbits based on electrophysiologic studies. Full thickness retinal penetration may explain observed clinical effects of intravitreal bevacizumab. Although it is difficult to directly extrapolate to humans, our study supports the safe use of intravitreal bevacizumab injection.

RETINA X:1-1, 2006

Vascular endothelial growth factor (VEGF) has been implicated as the major angiogenic stimulus responsible for the formation of choroidal neovascularization (CNV) in age-related macular degeneration (ARMD).[1] Drugs that inhibit the bioactivity of VEGF represent a new paradigm in the treatment of neovascular ARMD. Pegaptanib (Macugen; Eyetech Pharmaceuticals, Inc., New York, NY), an anti-VEGF aptamer, was found to be superior to sham injection in phase III trials and was the first anti-VEGF drug to be approved for the treatment of neovascular ARMD.[2] However, its advantage was manifested mainly by slowing the pro-

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The research was partially supported by the Selma Mitrani Fund for Research in Age Related Macular Degeneration (to I.P.). The research was performed in partial fulfillment of the MD thesis requirements of Yonathan Shahar, BSc, in the Sackler Faculty of Medicine, Tel Aviv.

None of the authors has a proprietary interest. Dr. Avery has received consulting fees from Genentech; however, Genentech did not sponsor this research.

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gression of vision loss rather than by improvement of visual acuity.[2]

Bevacizumab (Avastin; Genentech Inc., San Francisco, CA) is a Food and Drug Administration–approved anti-angiogenesis drug that is given intravenously in combination with 5-fluorouracil (5-FU) for treating advanced colorectal cancer.[3] It is a humanized monoclonal antibody that binds all isoforms of VEGF ($VEGF_{110,}$ $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$). Recently, it has been demonstrated that systemic administration of bevacizumab is effective in patients with neovascular ARMD, improving visual acuity and reducing retinal thickness.[4] However, systemic administration of bevacizumab has been associated with an increased risk of thromboembolic events in cancer patients.[5] Ranibizumab (Lucentis, Genentech Inc.) is a smaller 48-kD Fab fragment derived from the same murine antibody as bevacizumab.[6] In a large prospective study, Ranibizumab showed promising results, including improvement in visual acuity to 20/40 in approximately 40% of treated ARMD patients; furthermore, visual acuity improved by 3 lines in 30% of patients, with a mean increase of 7 letters (Miller JW, ASRS meeting, July 2005, Montreal).

Ranibizumab was developed, in part, because preclinical primate data reportedly showed lack of penetration of intravitreally injected full length IgG beyond the internal limiting membrane (ILM).[7] However, in clinical practice, intravitreal bevacizumab has been found to have a significant biologic effect on retinal edema, subretinal fluid, and pigment epithelial detachments secondary to ARMD[8] (Rosenfeld PJ, Avery RL, Subspecialty Retina Meeting, AAO, October 2005; Chicago) as well as reducing the macular edema of vein occlusions.[9] Because of this disparity, we decided to evaluate the retinal penetration of bevacizumab following intravitreal injection in rabbits.

A further aim of the present study was to evaluate possible toxicity of intravitreally injected bevacizumab in a rabbit model. To test possible bevacizumab toxicity to the ganglion cells as well as to distal retinal layers, we recorded respectively the visual evoked potentials (VEPs) and the electroretinogram (ERG).

Methods

Animals

Ten adult albino rabbits weighing 2.5–3.0 kg each were included in the study. The rabbits were housed under 12/12 hour light-dark cycle and were allowed free access to water and food. All the experimental procedures adhered to the ARVO Resolution on the Treatment of Animals in Research and to institutional guidelines. Before intravitreal injection and electrophysiologic recordings, the rabbits were anesthetized by an intramuscular injection (0.5 mL/kg body weight) of a mixture containing ketamine hydrochloride (10 mg/mL), acepromazine maleate solution (10%), and xylazine solution (2%) at a ratio of 1:0.2:0.3. Topical anesthesia (benoxinate HCL 0.4%) was administered to reduce the animals' discomfort. The pupils were fully dilated with cyclopentolate hydrochloride 1%. The rabbits underwent clinical inspection by indirect ophthalmoscopy, ERG, and VEP recordings. The ERG responses were recorded from each rabbit before intravitreal injection, 3 hours postinjection to detect immediate functional damage to the retina, and then 3 days and 1, 2, and 4 weeks postinjection to determine possible permanent damage to the retina. Four weeks after injection, the visual evoked potentials were recorded as well. Then, the rabbits were killed by intravenous injection of an overdose of sodium pentobarbital (80 mg/kg body weight).

Bevacizumab (Avastin)

The right eye of each rabbit was injected intravitreally with 0.1 mL bevacizumab solution having a concentration of 25 mg/mL (original vial concentration).

Intravitreal Injection

Each rabbit was injected intravitreally in both eyes as described before.[10] A 28-gauge needle attached to a 1.0-mL tuberculin syringe was inserted into the vitreous approximately 1 mm posterior to the limbus. The syringe was directed under visual control using an indirect ophthalmoscope (Neitz Instruments, Tokyo, Japan) towards the center of the vitreous above the optic disk. A volume of 0.1 mL was then slowly injected. The right eye was always injected with bevacizumab, and is referred to as the experimental eye. The left eye of each rabbit was injected intravitreally with 0.1 mL PBS and served as control.

Following the intravitreal injection the rabbit underwent an ophthalmoscopic examination for detection of retinal injury or cataract formation.

Electroretinogram

Flash ERG responses were recorded[10] from the experimental and control eyes, using corneal electrodes (Medical Workshop, Groningen, The Netherlands). The reference and ground electrodes were made of stainless steel surgical needles, and were

inserted into the ears. The ERG signals were amplified (x20,000) and filtered (0.3–300 Hz) by differential amplifiers (Grass, West Warwick, RI). Light stimuli were obtained from a Ganzfeld light source (LKC Technologies, Gaithersburg, MD) with a maximum intensity of 5.76 cd-s/m^2.

The ERG responses were recorded simultaneously in the dark-adapted state (at least 3 hours in darkness), and then in a light-adapted state (background illumination of 1.15 cd/m^2). Six responses elicited by identical flashes applied at 10-second intervals, were averaged in the dark-adapted state, and 15 responses elicited at a frequency of 1 Hz were averaged in the light-adapted state.

ERG analysis was based on measurements of the b-wave amplitude from the trough of the a-wave to the peak of the b-wave. The b-waves of the experimental and control eyes were plotted as a function of log flash intensity for each eye of each rabbit. The response-intensity curve of the ERG b-wave was fitted to a Michaelis-Menten type hyperbolic function[11]:

$$V/Vmax = I/(I + \sigma) \quad (1)$$

where V is the amplitude of the ERG b-wave elicited by a stimulus of intensity I, Vmax is the maximal response amplitude and σ is the semi-saturation constant. Functional damage in the experimental eye was assessed from the Vmax ratio (experimental/control), and the difference in logσ (experimental–control).

Visual Evoked Potentials

Flash VEP was recorded using a stainless steel needle as the active electrode that was inserted under the skin above the area of the visual cortex, midway between the two ears. The reference and ground electrodes were inserted in the ears. The signal was amplified (x200,000) and filtered (1–100 Hz) by a differential amplifier (Grass, West Warwick, RI). With this electrode configuration, monocular light stimuli yielded very similar VEPs in nontreated animals. Fifty stimuli were delivered at a rate of 1.1 Hz, and the resultant signals were digitized and averaged by the computer.

The VEP responses were assessed quantitatively from their temporal pattern and amplitudes. The most easily identifiable waves of the flash VEP in rabbits were an initial negative wave that was followed by a prominent positive wave. The VEP amplitude was measured from the trough of the first negative wave to the peak of the following positive wave. Temporal properties of the VEP were defined by the time interval from stimulus onset to the trough of the first negative wave, termed the implicit time.

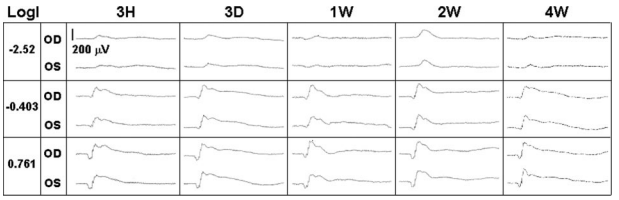


Fig. 1. Electroretinogram (ERG) follow-up of one albino rabbit that was injected with 2.5 mg of bevacizumab. Dark-adapted ERG responses were elicited by light stimuli of different intensities as denoted in log units of cd-s/m2 to the left of each row of responses. ERG responses were recorded 3 hours, 3 days, 1 week, 2 weeks, and 4 weeks after injection. Each pair of responses compares the ERG of the experimental eye to that of the control eye (upper and lower traces, respectively). Calibration bars: vertical 100 μV, horizontal 100 msec.

Immunohistochemistry

Following fixation for at least 24 hours, 4 mm square pieces of retina attached to the retinal pigment epithelium (RPE) were excised from the eye and rinsed in phosphate buffered saline (PBS, pH = 7.2). The tissue was then embedded in low-melt agarose (5%; Sigma, St. Louis, MO) and sectioned at 100 μm with a Vibratome (Technical Products International, Polysciences, Warrington, PA). Sections were incubated in normal donkey serum (1:20) overnight in PBS, 0.5% BSA, 0.1% Triton X-100, and 0.1% azide (PBTA). The next day the donkey anti-human antibody conjugated to the fluorochrome Cy3 (1:500 in PBTA; Jackson ImmunoResearch Labs, West Grove, PA) was added overnight. This antibody binds to the heavy and light chains of the humanized bevacizumab IgG antibody. All antibody incubations were done at 4°C on a rotator. The sections were then rinsed in PBTA, mounted on glass slides using 5% n-propyl gallate in glycerol, and viewed on an Olympus Flouview laser scanning confocal microscope.

Results

Clinical Observation

After intravitreal injection, as well as during the follow-up period, no inflammation was observed in any eye. In all eyes injected, the cornea was clear, there was no inflammatory response, the lens and vitreous appeared clear and the fundus intact in both the bevacizumab and the saline injected eyes.

Electroretinogram

Figure 1 shows representative ERG responses of one rabbit that were recorded at five time intervals (3 hours, 3 days, and 1, 2, and 4 weeks) after intravitreal injection of 0.1 mL of the original 25 mg/mL vial solution, namely, an injected bevacizumab dose of 2.5

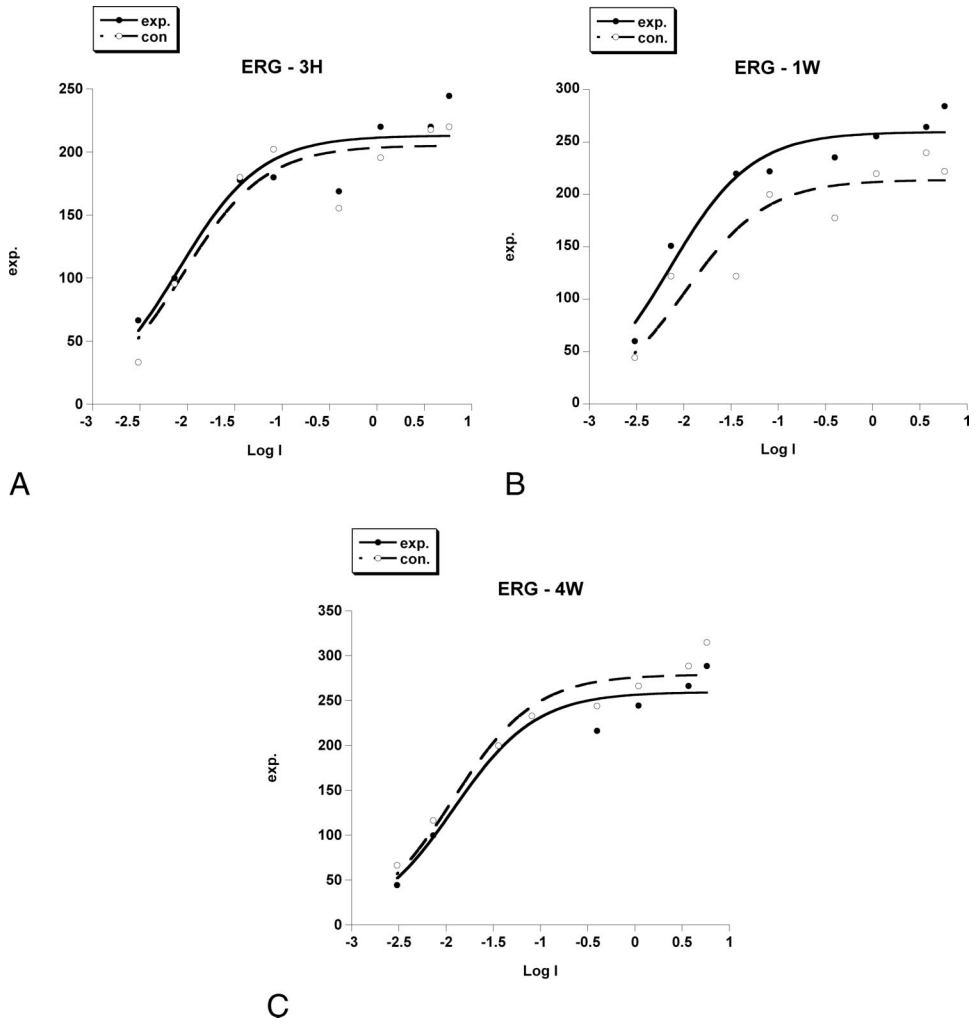


Fig. 2. Response-intensity data for the dark-adapted electroretinogram (ERG) b-wave were measured for the rabbit whose ERG responses are shown in Figure 1. ERG data of the experimental and control eyes are compared (filled and open symbols, respectively). The data were fitted to a Michaelis-Menten hyperbolic relationship (equation 1) (dashed curve for the control eye and a continuous curve for the experimental eye).

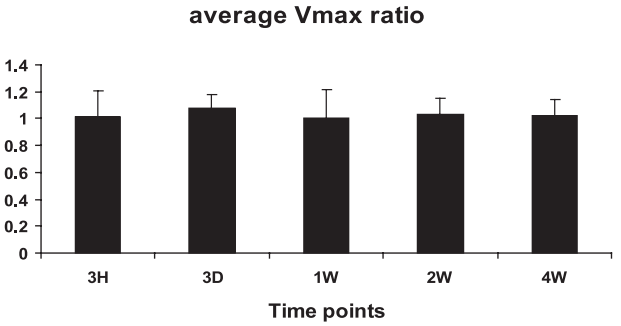
mg. Dark-adapted ERG responses that were elicited by flashes of different intensities (denoted in log units to the left of each row) are shown for each recording session. The ERG response of the experimental eye (upper trace) is compared to the ERG response of the control eye (lower trace) in each pair of responses. The ERG responses of the rabbit in Figure 1 did not change appreciably during the 4-week follow-up period.

The pattern of the bevacizumab effect and its dependency upon time after injection are better illustrated in Figure 2, showing the response-intensity relationships for the dark adapted b-waves measured at 3 hours (A), 1 week (B), and 4 weeks (C) after bevacizumab injection. The response-intensity data were fitted to the Michaelis-Menten hyperbolic function (equation 1) to derive the maximal b-wave amplitude (Vmax) and semi-saturation constant (σ), summarized in Table 1.

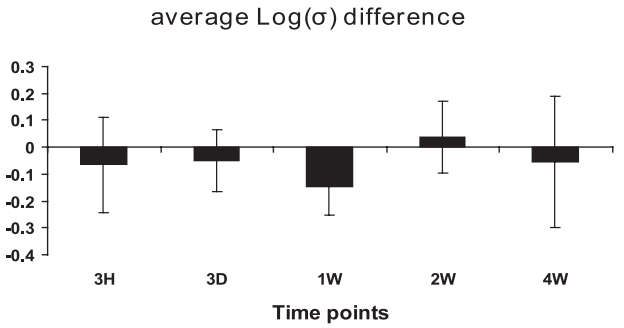
Table 1. Maximum b-Wave Amplitude (Vmax) and Semi-saturation Constant (σ) of the Rabbit, Whose Electroretinogram Responses and Response-Intensity Data are Shown in Figures 1 and 2, Respectively

	Vmax (exp)	Vmax (control)	Vmax ratio	Logσ (exp)	Logσ (control)	Δ Logσ
3 hours	213.08	205.21	1.038351	−2.0546	−2.0962	−0.0416
1 week	259.78	214.03	1.213755	−2.1494	−1.9904	−0.159
4 weeks	259.6	279.05	0.930299	−1.9194	−1.9299	0.0105

Bevacizumab toxicity was assessed from the Vmax ratio and the difference in logσ.
Vmax values are given in μV.
σ values are given in cd-s/m^2.



A



B

Fig. 3. Electroretinogram (ERG) follow-up for 10 albino rabbits. (A) Vmax ratio (experimental eye/control eye) was calculated for each rabbit at each ERG recording session. The Vmax ratios of rabbits were averaged for each testing session, to assess bevacizumab toxicity. (B) The effects of bevacizumab on the semi-saturation constant were calculated from the difference between logσ of the experimental eye and logσ of the control eye.

To asses the effects of bevacizumab on the functional integrity of the experimental eye, we calculated the Vmax ratio (experimental/control eye) and the semi-saturation constant (logσ difference) of b-waves in the dark-adapted state, as shown in Figure 3, A and B, respectively. For the light-adapted state we calculated the amplitude ratio for the ERG responses evoked by the brightest light flash. Figure 3A summarizes the average Vmax ratio of the rabbits at each postinjection time point. Figure 3B summarizes the average difference in logσ of the rabbits at each postinjection time point. There was no significant difference in the Vmax of b wave in dark adapted state and the logσ at all the different time intervals. Figure 4 shows the average amplitude ratio of b waves in the light adapted state at all the different time intervals. Here also there was no significant difference at the different time points.

Visual Evoked Potential

Since bevacizumab penetrated the retina from the vitreal side, the first cells to be exposed to it were the ganglion cells. We recorded the VEPs to test possible

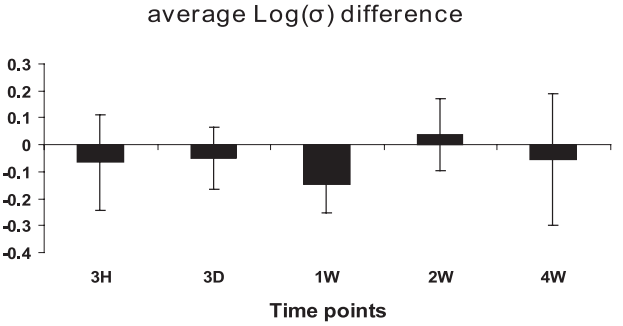


Fig. 4. Average ± SD amplitude ratio of b waves in the light-adapted state (background intensity of 1.15 cd/m^2 at all the different time intervals). Here also, there was no significant difference between the experimental and control eyes of all the recording sessions.

bevacizumab toxicity to the ganglion cells and/or to the nerve fiber layer.

Figure 5 shows VEP responses elicited by binocular stimulation (upper trace) and by monocular stimulation of the experimental and control eyes (middle and lower rows, respectively) of one rabbit that were recorded 4 weeks after intravitreal injection of bevacizumab. The typical pattern of a negative wave appearing 40 to 60 msec after the light stimulus, followed by a positive wave, was seen in all recordings for experimental and control eyes.

We measured the implicit time of the first negative wave and the amplitude of the positive wave of the VEP responses of all the rabbits, at the 4-week time point, and calculated the amplitude ratio (experimental/control) and the implicit time difference (experimental – control). The average amplitude ratio was 1.06 ± 0.27, and the average implicit time difference was 1.23 ± 5.45. These values did not differ significantly from a ratio of 1.0 and from a difference of 0 for the amplitude ratio and implicit time difference, respectively.

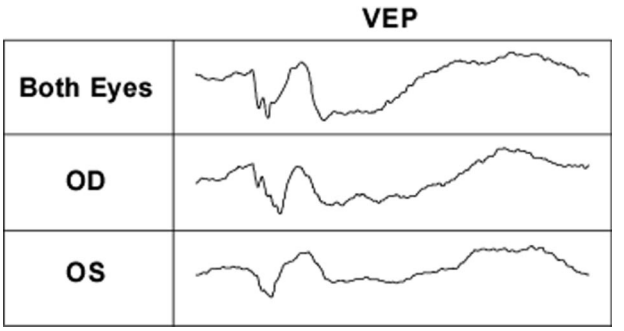


Fig. 5. Flash visual evoked potential (VEP) responses were elicited by bright white light stimuli. VEP responses elicited by binocular stimulation (upper trace) and by monocular stimulation of the experimental and control eyes (middle and lower rows, respectively) of one rabbit 4 weeks after intravitreal injection of 2.5 mg of bevacizumab.

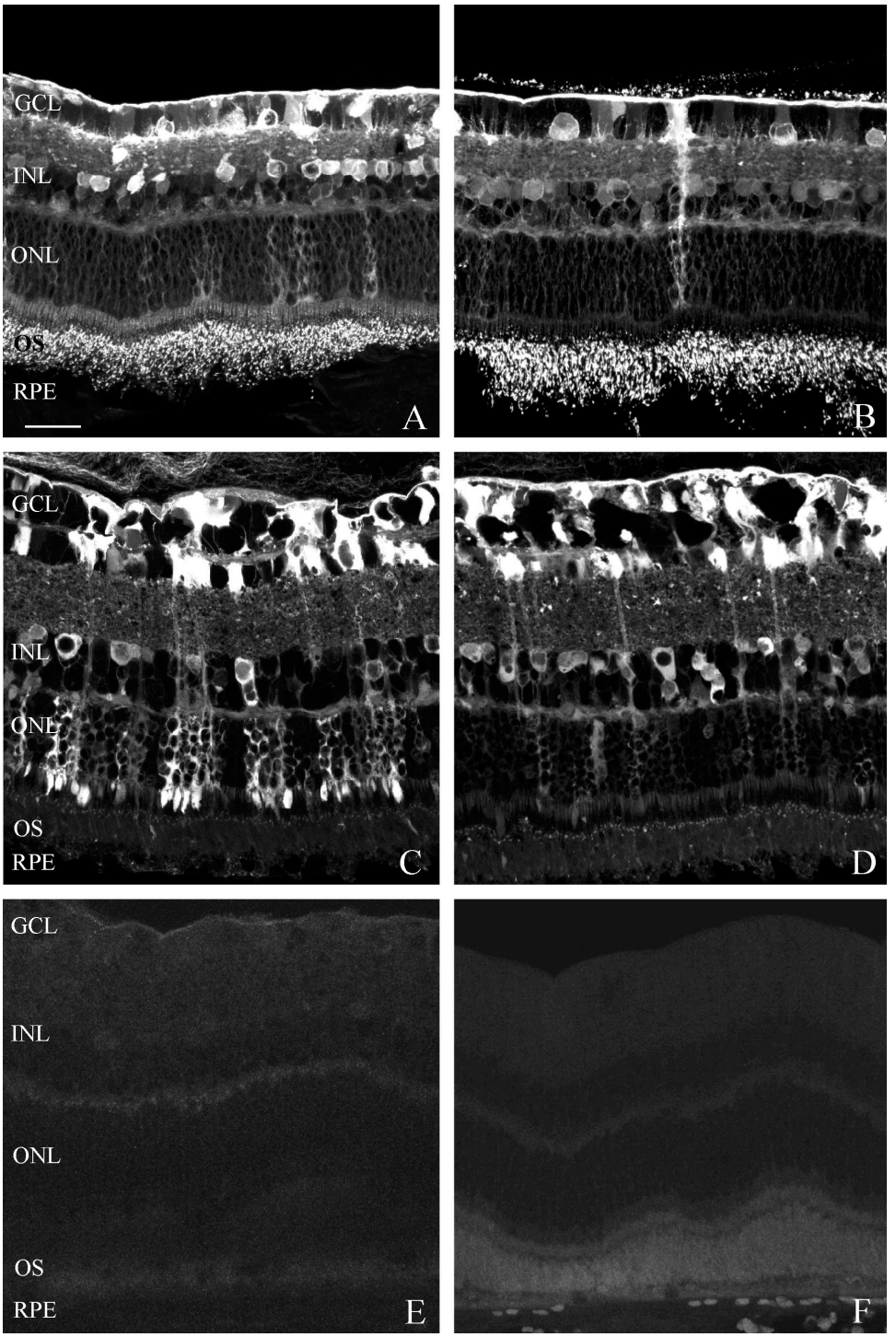


Fig. 6. Laser scanning confocal microscope images of eyes at 1 (**A, B**), 7 (**C, D**), and 28 (**E**) days after intravitreal injection of bevacizumab. **A, B,** 1 day. Antibody labeling was detected along the internal limiting membrane, in specific cells within the ganglion cell layer (GCL) and inner nuclear layer (INL), in Müller cells extending across the entire retina, and in the outer segment (OS) layer. **C, D,** 7 day. Intense labeling was observed in Müller cells at the end foot region in the GCL as well as in some regions of the ONL. Labeling was also observed in specific cells in the GCL and INL. No labeling was observed in the OS at this time. **E,** 28 day, high gain settings. Using significantly increased gain settings, only very slight ILM labeling could be observed over baseline autofluorescence. (No labeling or autofluorescence was observed using the same microscope settings as the 1 and 7 day time points.) No labeling was observed in the retinal pigment epithelium (RPE) or choroid at any time point. **F,** Control, 28 day, high gain settings. Autofluorescence was observed in retinal sections taken from the uninjected control eye and stained with the secondary antibody alone when scanned at the increased gain settings used in **E.** (No labeling or autofluorescence was observed using the same microscope settings as the 1 and 7 day time points.) Each image is a projection of 6 "z" images. Bar, 20 μm.

Immunohistochemistry

In the eyes that had been injected with bevacizumab 24 hours before being killed, labeling was observed throughout the retina but not within the RPE or choroid (Figure 6, A and B). Specifically, labeling was present in the ILM, the ganglion cell, and inner nuclear layer, as well as in the inner and outer segment layers of the photoreceptors. In some cases, entire Muller cells were labeled. While much of the retinal bevacizumab labeling was extracellular, in some cases it appears that this antibody had been internalized by a subset of cells within the retina. A similar pattern of

labeling was observed in eyes that had been injected with bevacizumab 7 days before being killed, although the intensity of Muller cell labeling appeared to increase and essentially no labeling was observed in the inner and outer segment layers (Figure 6, C and D). There was no labeling seen in saline injected eyes when the same microscope settings were used as those for the contralateral bevacizumab injected eyes (data not shown). In eyes that had been injected with bevacizumab 4 weeks before being killed, no labeling was observed when the sections were viewed using identical microscope gain settings as those used for the 24 hours and 7 day injected eyes. When the gain was increased significantly, faint labeling of the ILM was detectable (Figure 6E) above background autofluorescence which was seen in the uninjected control eyes that were incubated with the donkey antihuman Cy3 (Figure 6F).

Discussion

The finding of full thickness retinal penetration of this humanized IgG after intravitreal injection contradicts the findings of Mordenti et al., but may help explain the biologic effect of intravitreal bevacizumab seen clinically.[7]

There are several possible explanations for the different results obtained in our study and those obtained by the previous primate study. First, different species were used that may react differently to injected antibody. Other differences may also be important. For example, the previous study compared penetration differences between IgG and Fab fragments, and different epitopes were used.[7] The IgG used in the study was one made against human epidermal growth factor 2 (HER2), and the Fab fragment was made against VEGF. The retinal penetration may be different due to the binding of HER2 sites in the inner retina even though the administration of excess unlabeled anti-HER2 antibody in these experiments may have minimized this binding. Another reason may relate a difference in dosage. The previous study used a dose of 25 μg, while 2.5 mg was used in our study (which is closer to the 1.25 mg which is being used clinically in humans).[7] This 100-fold increase in dose may overwhelm barriers to diffusion, allowing a small amount of this potent agent to diffuse into the retina and exert a biologic effect. In fact, Han has used very high doses of IgG in rabbits to explore a therapeutic use for treating endophthalmitis. He found full thickness retinal penetration at all doses tested ranging from 0.5 to 30 mg.[12]

Another reason for the observed differences may be the different techniques used to evaluate retinal penetration. The confocal microscope utilizes fluorescently labeled probes, is a very sensitive technique, and employs higher resolution to localize bevacizumab binding to individual cells than tissue autoradiography. The localization of bevacizumab binding to Muller and ganglion cells in the current study is consistent with prior localization of VEGF and VEGF receptors.[13] In addition, some of the cellular labeling within the inner retina may represent labeling of microglia or other cells capable of binding the Fc portion of bevacizumab. It is possible that a more sensitive assay would be able to detect bevacizumab within the retina at longer time points after injection as its concentration decreases, but for proof of concept that bevacizumab penetrates the retina in this model, the assay certainly seems sensitive enough. There is strong, specific labeling in the bevacizumab injected eyes for at least 7 days, whereas the control images at the same gain settings are completely black, without any labeling.

Finally, the clinically observed biologic effect of intravitreal bevacizumab may relate to the anatomy of the human foveola. Green has written that the ILM is greatly attenuated or absent over the foveola.[14,15] Hogan et al state that its thickness drops to 10 to 20 nm in this region, whereas it is up to 40-fold thicker in the rest of the posterior pole.[16] In a primate study, the ILM thickness has been reported to be 30 nm at the foveal pit, but up to 2000 nm in the peripapillary area.[17] In addition, the retina is thinned at the foveola, and lacks an inner plexiform layer—a layer which has recently been shown to be a potential diffusion barrier to molecules of greater than 76 kD.[18] The attenuation of the ILM and the absence of an inner plexiform layer at the foveola may allow increased diffusion of bevacizumab in this region where it would be of greatest benefit in the treatment of ARMD.

The observation that there was less retinal labeling of bevacizumab at 7 days and no labeling by 28 days is expected given the half-life of an antibody in the vitreous cavity. Mordenti et al found no retinal labeling for the Fab fragment by 14 days as it had been cleared from the eye.[7] Direct comparison of the retinal penetration and affinity/binding of ranibizumab and bevacizumab in this model are subjects of future research, but this study shows that intravitreal bevacizumab can penetrate beyond the ILM into the retina.

The clinical observations did not show any inflammatory response in any injected eye. The lack of such response in the rabbit eye, which is much more prone to such inflammation than the human eye, may predict the lack of inflammation in human eyes as well. The electrophysiologic results demonstrate no functional damage to the retina of albino rabbits by intravitreal injection of bevacizumab. Even though only two case reports have been published thus far in the peer-reviewed ophthalmologic literature,[8,9] others are in

press,[19] and personal communication reveals widely spreading off-label use of intravitreal bevacizumab worldwide. Thus far, no toxicity studies have been reported to support the safety for such intravitreal treatment. Our findings provide support from animal models for the use of this potentially breakthrough therapeutic modality.

The use of intravitreal bevacizumab is potentially preferable to the use of intravenous bevacizumab. First, intravitreal delivery of bevacizumab should lower the risk of systemic adverse effects associated with intravenous injection. Secondly, the dose needed for intravitreal injection is approximately 400-fold smaller compared with an intravenous dose. This will significantly lower the cost of treatment from approximately $2,200 USD per patient (using four vials); the cost of a 1 mg intravitreal dose is only $5.5 USD.[20]

The use of intravitreal bevacizumab may also be more cost effective than other intravitreal antiangiogenic drugs such as pegaptanib and ranibizumab. It may potentially prove to be more efficacious than pegaptanib due to its ability to block all isoforms of VEGF as ranibizumab does. A potential advantage over ranibizumab is its predicted longer half life in the eye, thus requiring less frequent dosing.[6] It also may be an alternative in cases responding poorly to already approved treatment modalities for CNV.

Bevacizumab may prove desirable for primary treatment if future studies show better visual acuity results or the need for less frequent administration.

Based on electrophysiology, our study showed no evidence for retinal toxicity resulting from a single intravitreal injection of bevacizumab in a rabbit model. Extrapolation from animal to human studies should be done with caution. However, it should be noted that the dose injected in our study was twofold greater than that currently used in humans. Furthermore, the drug was injected into a smaller vitreous volume, less than 2 mL, in the rabbit as opposed to 4 to 5 mL in humans. Thus, our study supports the ocular safety of intravitreal bevacizumab injection.

Key words: Avastin, bevacizumab, intravitreal injection, retinal toxicity.

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