Penetration of Bevacizumab through the Retina after Intravitreal Injection in the Monkey

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PURPOSE. The penetration of intravitreally injected bevacizumab in its commercial formulation (Avastin; Roche, Grenzach, Germany) through the retina was studied, to determine whether a full-length antibody would be able to penetrate the retina as easily as an antibody fragment.

METHODS. Six cynomolgus monkeys (Macaca fascicularis) were used in this study. Two compositions of intravitreal injection into the right eyes were performed: one with commercial Avastin (group 1, four animals) and the other one with commercial Avastin labeled with 125I (group 2, one animal). The animals in group 1 were killed 1, 4, 7, or 14 days after the injection for subsequent histologic analysis of the eyes by immunohistochemistry, and the animal in group 2 was killed 7 days after injection for autoradiography and electron microscopy. Funduscopy was performed before the injection and at several time points thereafter. Moreover, blood samples were collected at different time points from the group-2 animal. The sixth animal remained untreated and served as the control.

RESULTS. No pathologic changes were obvious in the funduscopic images within the time of the experiment. Bevacizumab immunoreactivity was found in the choroid and the inner layers of the retina as early as 1 day after the injection and spread to the outer layers and the choroid within the following days, in particular to photoreceptors and blood vessels. Avastin labeled with 125I showed radioactivity in blood serum 1 day after the intravitreal injection and remained relatively stable until day 7.

CONCLUSIONS. The results clearly show that the bevacizumab molecule can penetrate the retina and is also transported into the retinal pigment epithelium, the choroid and, in particular, into photoreceptor outer segments after intravitreal injection of Avastin. Active transport mechanisms seem to be involved.

Nevascularization occurs in several diseases of the eye, such as wet age-related macular degeneration, proliferative diabetic retinopathy, ruberosis, or retinopathy of prematurity. One of the major factors inducing formation of new vessels is vascular endothelial growth factor (VEGF), acting via its corresponding receptors during embryonic development and in eye diseases.

During the past several years, inhibition of VEGF and its receptors has drawn much attention. Two main VEGF inhibitors have been launched recently, pegaptanib (Macugen; Eye Tech/Pfizer, New York, NY) and ranibizumab (rhuFab V2; Lucentis, Novartis, Basel, Switzerland), with promising results in clinical trials, in particular for ranibizumab.

Another VEGF-binding compound is bevacizumab (Avastin; Roche, Grenzach, Germany), which is a complete humanized murine monoclonal antibody against all isoforms of human VEGF. Bevacizumab has recently been approved by the U.S. Food and Drug Administration for the treatment of metastatic colorectal cancer and is in Phase III trials for advanced breast and renal cancers. Compared to the other VEGF-inhibitors just mentioned, bevacizumab is by far the less cost-intensive treatment when injected intravitreally, which is important considering the reinjections that are necessary at 4- to 6-week intervals. Systemically or intravitreally applied bevacizumab significantly reduced macular edema in patients with ARMD and central vein occlusion. However, when used in cancer treatment and applied intravenously, bevacizumab caused adverse events in patients in some cases, such as hypertension, neuropathy, congestive heart failure, hemorrhage and bleeding, neutropenic complications, proteinuria, and arterial thromboembolic events. Therefore, intravitreal (i.e., local) administration appears to be favorable, because relatively high concentrations of the bevacizumab protein may be applied locally. In addition, as the eye is a relatively closed compartment, it may be anticipated that bevacizumab would not pass into the blood circulation to a very great extent, which would further reduce the risk of side effects.

The purpose of this work was to study the penetration of intravitreally injected bevacizumab in its commercial formulation (Avastin; Roche) through the retina, since whether a full-length antibody would be able to penetrate the retina as easily as an antibody fragment is still up for speculation. Moreover, a first approach was tried to find out what cell types eventually take up and/or transport the bevacizumab molecule.

METHODS

Animals

Six cynomolgus monkeys (Macaca fascicularis) were used (8–9 years old, 2.9 to 3.7 kg, supplied by Nafovanny, Long Thanh, Vietnam). They were housed in the animal facilities of Covance Laboratories (Münster, Germany) under standard conditions. Before the experiments were started, animals were approved as experimental animals by a veteri-
narian of the local authorities and the site veterinarian. The animal experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Short Study Protocol**

Two types of intravitreal injection were performed: one with commercial Avastin (group 1, four animals) and the other with commercial Avastin labeled with 125I (group 2, one animal). The four animals in group 1 were killed 1, 4, 7, or 14 days after injection for subsequent analysis of the right eyes by immunohistochemistry. The group 2 animal was killed 7 days after injection for subsequent analysis of the right eye by autoradiography and electron microscopy. Funduscopy was performed in all animals before the injection and at several time points thereafter. Moreover, blood samples were collected at different time points from the group-2 animal that received radioactively labeled protein. A sixth animal of the same origin that remained completely untreated was used as a control. The left eyes of all animals were processed for electron microscopy for purposes not related to this study.

**Fundus Photography**

Animals were lightly sedated with ketamine hydrochloride (Ketavet; Pharmacia GmbH, Erlangen, Germany). A mydriatic agent (tropicamide) was instilled in the eyes before examination. Fundus photographs were obtained in all animals once during the pre-dose phase and on the day before necropsy. A local ophthalmic anesthetic (proxymetacaine; Proparakanin-pos 0.5%; Ursapharm, Saarbrücken, Germany) was instilled in both eyes of each animal before fundus photography. The equipment used was a digital stationary fundus camera (TRC-50 ex; Topcon, Tokyo, Japan).

**Blood Sample Collection and Processing**

Blood samples (approximately 2 mL into EDTA tubes) were withdrawn from the animal injected with 125I-labeled bevacizumab on days 1, 4, and 7 after injection and were processed to plasma to evaluate the radioactivity. Samples were collected from the saphenous, brachial, cubital, or femoral veins and centrifuged for 10 minutes at 1000g. The supernatant was withdrawn and stored deep-frozen at −20 ± 4°C until determination of radioactivity.

**Labeling of Bevacizumab and Determination of Radioactivity**

Labeling of the bevacizumab protein with 125I was performed by Amersham Biosciences UK Ltd. (now GE Healthcare, Little Chalfont, UK), according to a method described previously. The activity of the final product was 0.87 kBq/ng (0.024 μCi/ng), and a total activity of 250 kBq (6.8 μCi) was injected into the eye of the monkey. Radioactivity of the blood samples was determined with a luminometer (Hidex; Triathlon, Turku, Finland) capable of gamma counting. The samples were instilled into a hole in a 1.25 × 1.25-in. sodium iodide crystal, and radioactivity was measured over 100 seconds. Calibration was performed by measurement of a sample with known activity. The values were corrected by the radioactivity of the empty NaI crystal as well as the decay of activity due to the 125I half-life of 90 days.

**Intravitreal Injection**

Animals were sedated by intramuscular injection of a mixture of ketamine hydrochloride (10 mg/kg) and xylazine (2 mg/kg). Intravitreal injections were performed in the right eyes of all animals. After inspection and examination of the eye and lids, a topical anesthesia was applied over the injection site using oxybuprocaine hydrochloride. The conjunctival and corneal surfaces were then irrigated with povidone iodine (10%) for 3 minutes. The eye was covered with sterile coatings, and a lid speculum was placed. Commercial Avastin (solution of bevacizumab, 1.25 mg in a volume of 50 μL) was placed under sterile conditions in a 1-mL syringe by the Department of Pharmacy, University Hospital of Tübingen. With a 27-gauge needle, 50 μL bevacizumab solution was injected 2 mm posterior to the corneal limbus into the vitreous cavity. The injection site was compressed for a minute using a forceps to avoid reflux on removal of the needle. Topical antibiotic ointment was administered four times daily for 4 days.

**Immunohistochemistry**

The right eyes taken from the group-1 animals where Avastin had been injected were fixed in formalin, embedded in paraffin wax, cut into 5-μm sections and deparaffinized according to standard procedures. Donkey anti-human IgG labeled with Cy3 (709-166-149, dilution 1:500; Jackson Immunoresearch, West Grove, PA) was used for bevacizumab IR detection. This polyclonal antibody binds to many epitopes of both Fc and Fab portions of human IgG. The following other antibodies were used for the detections of IR: rabbit anti-cow antibody (Z 334, dilution 1:4000; Dako, Carpinteria, CA) for glial acidic fibrillary protein (GFAP) IR, with an anti-rabbit IgG labeled with FITC (F9511, dilution 1:100; Sigma-Aldrich, St. Louis, MO) as a secondary antibody; mouse antibody (M7025, dilution 1:400; Dako) for vimentin IR, with goat anti-mouse IgG labeled with Alexa488 (A11001, dilution 1:400; Invitrogen-Molecular Probes, Eugene, OR) as a secondary antibody; a mouse antibody (VEG F C-1; sc-7269, dilution 1:400; Invitrogen-Molecular Probes) for angiopoietin (Ang)-2 IR, with goat anti-mouse antibody labeled with Alexa488 (A11001, dilution 1:400; Molecular Probes) as a secondary antibody. Stained retinal sections were embedded (FluorSave; Calbiochem, La Jolla, CA) and inspected with a fluorescence microscope or a confocal laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany).

**Electron Microscopy**

For electron microscopic investigation, the right enucleated eye from the group-2 animal with radioactively labeled bevacizumab was used and processed as follows. After removal of the cornea, the eye was fixed overnight at 4°C in glutaraldehyde (2% solution in 0.1 M cacodylate buffer [pH 7.4]). Half of the fixed eye was used for autoradiography as described later and was therefore embedded in paraffin wax using standard procedures. The other half eye was processed further for electron microscopy as follows. After washing with cacodylate buffer, areas of interest in flatmount preparations were excised and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer at room temperature for 1 hour. Dehydration was then started by a series of incubations in 30%, 50%, and 70% ethanol (10 minutes each). The samples were stained with saturated uranyl acetate. Dehydration was continued by incubations in 70%, 80%, and 96% ethanol (10 minutes each), absolute ethanol (two times for 15 minutes each), and propylene oxide (two times for 15 minutes each). The samples were then embedded in Epon. Ultrathin sections (70 nm) were made and stained with uranyl acetate and lead citrate and observed under an electron microscope (model 902 A; Carl Zeiss).

**Autoradiography**

Paraffin-embedded sections obtained from the half eye of the animal that received radioactively labeled protein were mounted on glass slides and coated with photographic emulsion (NBT-3; Eastman Kodak, Rochester, NY). The photographic emulsion was exposed to the sections for 3 to 4 weeks and was then developed (D-19 developer; Eastman Kodak), fixed (Rapidfix; Eastman Kodak), and inspected with a light microscope.

**Results**

**Funduscopy**

Funduscopic images were taken of each animal before the injection of Avastin and at different time points thereafter, depending on the duration of the experiment.
No obvious changes were found funduscopically in the eyes after the injections at the different time points (Fig. 1). In the one animal killed after the first day, the macula of the right eye was not recognizable. Histologic inspection of this eye showed a normal, intact macula, and we could not determine which alterations of the vitreous or the cornea caused the recognizability of the macula to be compromised.

**Immunohistochemistry**

Retinal slices prepared at different time points after the injection of Avastin were checked for their IR against the bevacizumab molecule (Fig. 2).

One day after the injection, bevacizumab IR was found in the inner limiting membrane (ILM); the ganglion cell layer (GCL); and, to a lesser degree, the inner plexiform layer (IPL; Fig. 2A). The ILM was found to be detached from the retina at several places, and the GCL appeared to be loosened.

Four days after injection, the inner retinal layers still showed IR for bevacizumab, though less intensive, with a faint staining also in the IPL and single cells in the inner nuclear layer (INL; neurons and/or Müller cells) and the outer plexiform layer (OPL). It appeared that the staining was present mainly on the surface or inside the processes of the cells (Fig. 2B).

The ILM was still stained 7 days after the injection of Avastin (Fig. 2C). A faint staining was also visible in the inner retinal layers. Most striking, there was a prominent staining of the photoreceptor (PR) outer segments (OS).

PR OS and the ILM were also stained 14 days after the injection (Fig. 2D). In addition, some PR inner segments (IS) were stained, as were clublike structures located near the OLM. Endothelial cells of the retinal blood vessels also exhibited bevacizumab IR.

Strong staining of choroidal blood vessels was seen at all four investigated time points, as shown in Figures 2A–D. In some places, the vessels were filled with material showing bevacizumab IR (Fig. 2B, arrow).

No specific staining was found when Cy-3-conjugated streptavidin was applied to a retinal section obtained 14 days

![Figure 1. Typical fundus photographs obtained in a monkey before and after the injection of Avastin in the right eye. The bright structures appearing in several images near the blood vessels are most probably light reflections.](image-url)
after the injection of Avastin (Fig. 2E) or when the anti-IgG antibody was applied to a retinal section from the control animal that did not receive Avastin (Fig. 2F). This demonstrates that the anti-IgG antibody used in our study indeed recognizes the injected bevacizumab molecule and that there is no non-specific fluorescence of the retinal sections except in the RPE, where it is most probably caused by lipofuscin.

The same pattern of staining was found in the region of the macula. As an example, the macular region 1 day after the injection is shown in Figure 3. Bevacizumab IR is visible in the ILM, the GCL, and a part of the INL. Again, the choroid is stained noticeably.

As mentioned already, the inner part of the retina showed some distortions 1 day after intravitreal injection of Avastin. The distortion can also be seen in Figure 4 in sections on which double staining against bevacizumab (red) and vimentin (green) had been performed. Figures 4A–C show different states of the retina, ranging from completely detached ILM and heavily loosened GCL to intact ILM. Several processes showing vimentin IR are seen that reach the plane of the ILM. Some of these processes show clear double staining (yellow appearance). Moreover, there are single round structures, probably ganglion cells, showing bevacizumab IR. In Figure 4D, a higher magnification of such a round cell is shown. It can be seen that the cell is surrounded by various processes that show vimentin IR. However, there is no bevacizumab IR detectable in the vimentin-positive processes.

Double staining of the retina against bevacizumab and GFAP 4 days after the injection did not yield uniform results throughout the retina. In several places, only minor expression of GFAP was seen (Fig. 5A). Bevacizumab IR was present in retinal layers from the ILM to the OPL, with predominance in the IPL and a subpopulation of cells in the INL (neuron and/or Müller cells). The radial orientation of stained processes between the ILM and INL suggests that these structures are Müller cells. There were also cells in the INL with radially oriented processes. The nature of these cells should be clarified by further investigation (i.e., whether they cells are Müller or bipolar cells).

A different situation is shown in Figure 5B. Müller cells were much more activated, as can be seen by the green fluorescence. There was also strong bevacizumab IR in the ILM and the IPL. In some rare cases, colocalization of GFAP and bevacizumab IR was observed, indicating the presence of bevacizumab in activated Müller cell processes.

Locally different states of glial activation, as indicated by different GFAP immunoreactivity, were found throughout the whole retina. Moreover, some cells immunoreactive for bevacizumab had a microglia-like morphology (Fig. 5; asterisks). On day 7 after the injection, bevacizumab IR (red) was found, not only in the PR OS, but also appearing as small stained dots in the RPE (Fig. 6, red arrows). Most probably, these dots represent phagosomes resulting from the phagocytosis of PR OS that contained bevacizumab before.

Bevacizumab–vimentin double staining was also performed in tissue samples obtained from eyes 14 days after intravitreal injection of Avastin (Fig. 7). As before, bevacizumab IR was present in the PR IS. As expected from the retinal structure, vimentin IR typical of Müller cells was visible in a fiberlike form within the ONL and stopped abruptly at the OLM (white arrows). Near the OLM, the vimentin-positive processes...
formed a basketlike structure. Of interest, bevacizumab IR was particularly high within these “baskets.”

Bevacizumab–rhodopsin double staining was performed in tissue samples from eyes 14 days after the intravitreal injection of Avastin (Fig. 8). Rhodopsin IR was seen in PR OS, with decreasing intensity toward the RPE (Fig. 8A). In contrast, bevacizumab IR was seen faintly in the PR IS, getting stronger in the PR OS. Dotlike rhodopsin IR was seen in the RPE, most likely representing rhodopsin from phagocytosed rod OS, which had not yet degraded. Strong bevacizumab staining was again observed in the blood vessel walls of the choroids (Fig. 8B). Moreover, there were several small structures in the lumen of the blood vessels showing strong bevacizumab IR (Fig. 8B, arrowheads).

Overlays of VEGF (green) and bevacizumab (red) IR are shown in Figure 9. In the control eye, VEGF immunoreactivity (IR) was visible in particular in the GCL and INL and, to a lesser degree, in the PRs and the choroidal blood vessel walls (Fig. 9A). One day after injection of Avastin, there was strong bevacizumab IR in the GCL and IPL as has been described (Fig. 9B). In contrast to the control eye, there was almost no VEGF IR left in the GCL. In some cases, VEGF and bevacizumab IR showed colocalization appearing as a yellowish stain. Visible VEGF IR was also diminished in the choroid. It remained visible in the vicinity of the choriocapillaris and was absent in other regions. There was colocalization of VEGF and bevacizumab IR in the blood vessel walls in some places.

Fourteen days after injection of Avastin, bevacizumab IR was present in the PR OS, the choroidal blood vessels, and the ILM. VEGF IR was almost completely depleted from the retina; the green fluorescence was near background levels. VEGF IR was still visible in some parts of the choriocapillaris in the choroid; however, it mainly colocalized with bevacizumab IR. In several arterioles of the choroid, a triple-layered structure of the vessel walls was recognizable (Fig. 10), with pure bevacizumab IR in the inner layer demarcating the vascular lumen (probably the intima), colocalized VEGF IR and bevacizumab IR in the outer layer (probably the adventitia), and more or less pure VEGF IR in the intermediate layer (probably the media).

**Electron Microscopy**

To get more information about the structures inside the “baskets” shown in Figure 7 as well as in outlines in Figure 1 (7 and 14 days, respectively, after injection), they were inspected by electron microscopy. Indeed, lengthy structures were found, one of them shown in Figure 11 approaching the OLM (black arrows) and extending into the ONL between the PR cells. Most probably, this is the transition from the nucleus to the IS of a rod.
Figure 12 shows a view of Bruch’s membrane. Basal membranes of endothelial and pigment epithelium cells are easily visible. There are some fenestrations (arrows) within the endothelial cells, which appear to be normal in structure.

Autoradiography

Bevacizumab was labeled with $^{125}$I and injected into the right eye of a monkey. The animal was killed and the eyes enucleated on day 7 after the injection. Radioactive labeling was found in all layers of the retina, including the RPE and the choroid (Fig. 13A). In particular, radioactivity was present in the OPL and the choroid. Sometimes, dark dots (silver grains) indicating radioactivity seemed to be arranged in lines through the inner retina, which could be an indication that bevacizumab was present in the Müller cells (Fig. 13A, red triangles).

Figure 13B shows a cross-section through a retinal blood vessel. Radioactivity appeared to be accumulated in the vessel wall, confirming corresponding observations made by immunohistochemistry.

The radioactivity of the blood samples that were taken from the monkey on days 1, 4, and 7 was measured after the intravitreal injection of bevacizumab labeled with $^{125}$I. The total amount of labeled protein in the blood was calculated taking consideration of the half-life of the isotope $^{125}$I. The results are shown in Figure 14. One day after intravitreal injection of radioactively labeled bevacizumab, radioactivity was detectable in the serum samples, which was equivalent to approximately 4% of the totally injected amount of radioactive protein. On days 4 and 7 after the injection, the percentage increased slightly to 5%.

Discussion

In the present study, we demonstrated penetration of bevacizumab through the monkey retina after the intravitreal injection of Avastin. To our knowledge, this is the first time that penetration of bevacizumab through the retina of monkeys has been demonstrated. As seen in Figure 2, penetration shows a time-dependent pattern. Bevacizumab IR was first detectable in the inner retinal layers and then spread to the outer layers of the retina. Throughout the whole time of the experiment (i.e., until day 14 after intravitreal injection), the ILM showed IR for bevacizumab, whereas the initially strong staining of the inner retinal layers disappeared 1 week after the injection, as though bevacizumab had been diluted thoroughly on its way through the retina. At later time points, bevacizumab IR was found predominantly in PR OS, as if it was “enriched” in these cells. These findings demonstrate that the bevacizumab molecule is well capable of penetrating the retina to reach its site of action, as may be deduced from the good clinical results reported after intravitreal injection of Avastin in patients.

The controls (Figs. 2E, 2F, 9A) showed that the antibody used for the detection of bevacizumab recognizes the injected bevacizumab molecule specifically and that the observed fluo-
Frescence does not arise from any other sources except the lipofuscin signal in the RPE.

The thickness of the ILM did not seem to play a crucial role in the velocity of retinal penetration of bevacizumab. No enhanced bevacizumab IR was observed in the region of the macula compared with other parts of the retina, as indicated in Figure 3.

In the autoradiographic images, a similar distribution of bevacizumab was seen, as found in the immunohistochemically processed samples. In particular, enrichment of bevacizumab in the blood vessel walls and in the region of PR OS was confirmed. Dark dots indicating radioactivity and hence the presence of bevacizumab was sometimes seen aligned in faint lines crossing inner retinal layers (Fig. 13A; red arrowheads). It is quite possible that these lines indicate the presence of bevacizumab in Müller cells.

**FIGURE 9.** Double staining for bev-acizumab (red) and VEGF (green). Fluorescence micrographs were merged and overlaid from retinal sections. (A) Control animal, with VEGF IR in the GCL, INL, ONL, and PR IS and weak staining in the choriocapillaris and the adjacent region of the choroid. Bevacizumab IR was visible in the vitreous, the ILM, and the GCL, as well as in the deeper choroid. (B) One day after injection of Avastin, there was VEGF IR in the INL, ONL, and PR IS and weak staining in the choriocapillaris and the adjacent region of the choroid. Bevacizumab IR was visible in the vitreous, the ILM, and the GCL, as well as in the deeper choroid. (C) Fourteen days after injection of Avastin, there was almost no VEGF IR in the inner retina, and weak staining in the choriocapillaris and blood vessel walls, with occasional colocalization with bevacizumab IR present mainly in PR OS and the choroid. Scale bar, 50 μm.

**FIGURE 10.** Fluorescence micrograph of double staining against bevacizumab (red) and VEGF (green) of a retinal section 14 days after injection of Avastin showing an arteriole displaying a triple layering of the vessel wall. The three layers—intima, media, and adventitia—showed IR for bevacizumab, VEGF, and both bevacizumab and VEGF, respectively. Scale bar, 50 μm.

**FIGURE 11.** Electron micrograph of a monkey retina 7 days after intravitreal injection of Avastin taken near the OLM (arrows) and showing a lengthy structure between PR nuclei that was found to be immunoreactive for bevacizumab.
To determine whether bevacizumab diffuses through the retina or whether active transport is involved (e.g., by Müller cells), we performed double-staining of the retina samples with antibodies against GFAP or vimentin. One day after intravitreal injection of Avastin, colocalization of vimentin and bevacizumab IR was seen in some processes, which were directed toward the ILM and most probably belong to Müller cells (Fig. 4). However, no such colocalization was found at later time points (Fig. 7). The reason for this observation is not clear at the moment.

Of interest, the choroidal blood vessels were positive for bevacizumab throughout the whole time of the experiment, indicating a substantial transfer of bevacizumab into the blood circulation. Accordingly, measurement of radioactivity in serum samples that were taken on days 1, 4, and 7 after Avastin injection showed that a noticeable part of the radioactivity seemed to have crossed the blood–retinal barrier readily after the injection. Whether this happens by active transport (e.g., endocytosis by the microglia, astrocytes, and Müller cells) with subsequent delivery into the blood circulation remains to be clarified. A lot of bevacizumab IR was found in the blood vessel walls and even within the vessel lumen, which is in line with the fast appearance of noticeable radioactivity in the blood serum samples.

In conclusion, the bevacizumab molecule penetrates the retina and finally reaches the PR OS. The time dependency of bevacizumab IR distribution suggests that bevacizumab spreads from the inner retina to the OS, where it stays over a longer period, whereas almost no bevacizumab IR was seen in the other retinal layers except the ILM.

It can be speculated that, due to the rapid transfer of bevacizumab into the blood circulation, circulating bevacizumab could penetrate through the Bruch’s membrane and the RPE, to reach the PR OS. At the moment, we do not have a conclusive mechanism for such a hypothesis, and we did not observe signs of a damage of the Bruch’s membrane or the RPE layer. In contrast, the presence of radioactivity in all retinal layers on day 7 after the injection (Fig. 12A) supports the assumption that bevacizumab is transported directly from the vitreous through the inner retina into the PR. Therefore, the route by which the injected bevacizumab finally reaches the PR OS remains unknown.

Figure 9A shows the distribution of VEGF in the normal retina. The main staining is visible in the GCL and INL and is weaker in the PR layer and the choroid. Such a distribution is in accordance with the results of Famiglietti et al., who studied distribution of VEGF in human retina and found it in various retinal neurons, Müller cells, blood vessels, and even PR, though to a lesser extent. Unlike these investigators, we did not find signs of VEGF IR in Müller cells.
Again, there is almost no red staining in Figure 9A, indicating high specificity of our bevacizumab detection. One day after injection of Avastin, almost no VEGF IR was detectable in the GCL (Fig. 9B), and almost no VEGF IR was visible in the whole retina 14 days after the injection (Fig. 9C).

The lack of VEGF IR in the retina 14 days after the injection cannot be explained at the moment. One possible explanation would be that VEGF cannot be recognized any more by its antibody if bevacizumab has bound to it; however, in this case bevacizumab IR should be visible because the polyclonal anti-lgG antibody we used for bevacizumab detection recognizes multiple sites of the bevacizumab molecule. If the VEGF present formerly in the retinal neurons has been abolished by the binding of bevacizumab, then the question arises of why the retinal neurons did not produce new VEGF in the meantime, in that there should be enough time to do so during 14 days.

In contrast, VEGF IR was retained in the choriocapillaris and Bruch’s membrane on day 1 after injection and was also partly visible on day 14. Colocalization of VEGF IR and bevacizumab IR can be observed in particular in the walls of large choroidal blood vessels. Famiglietti et al.15 described in their study a double layering of VEGF IR in the walls of the largest blood vessels. We have seen triple-layered arteriolar walls in retinal sections from the eye 14 days after injection (Fig. 10). Such a finding could be explained by assuming that bevacizumab spreads into the choroid from the blood vessels, that VEGF is newly formed in the intermediate layer (media) at a sufficient rate, and that the VEGF-bevacizumab complex accumulates in the outer layer (adventitia)—the latter at least to a certain extent. If such an explanation is also valid in the inner retina, then it could be supposed that the retinal neurons are not able to produce enough VEGF to compensate for the loss caused by the injected bevacizumab.

Because of its structure, the retina is a challenging environment for the diffusion of larger molecules. This is particularly the case for the inner limiting membrane and the interphotoreceptor matrix, which are rich in glycosaminoglycans.16–19 There were only a few reports about retinal penetration of protein molecules after their intravitreal injection, which is surprising, because growth factors have been injected in the past extensively into the vitreous to achieve neuroprotection in the retina.

As an example, Kamei et al.20 reported that FITC-labeled tissue plasminogen activator (tPA; molecular mass, 70 kDa) did not penetrate the retina within 24 hours after intravitreal injection in rabbits, whereas rhodamine B isothiocyanate-labeled dextran (20 kDa) was found in all retinal layers at that time point.20 However, the authors did not postulate that the inability of tPA to penetrate the retina was due to a diffusion barrier to molecules of the size of tPA, because albumin, which has similar molecular mass (68 kDa), had been shown in a different study21 to reach the subretinal space from the vitreous within a few hours in a different study. Instead, they attributed the failure of tPA to penetrate the retina to specific binding to heparin and fibronectin, which may be caused by the negative charge of the FITC label.20

Recently, Kwan et al.22 reported the retinal penetration of tenecteplase, a tPA analogue, which had been injected intravitreally in pigs, through the sclera. In contrast to Kamei et al.,20 they did not label the injected protein with FITC, but used the neutral label Alexa488, which may explain the success of the penetration.

Antibodies against recoverin that were found in the sera of patients with cancer-associated retinopathy syndrome were injected into the vitreous of Lewis rats.23 Twenty-four hours later, they were detected in the retina, and PR and bipolar cells were found to undergo apoptotic death.

Last, an antibody isolated from patients with melanoma-associated retinopathy (MAR) was injected intravitreally in monkeys.24 A relatively rapid decrease was found in photopic b-wave amplitudes in electroretinographic measurements within 1 to 3 hours. Moreover, immunohistochemical inspection showed penetration of all retinal layers by the MAR IgG.

In contrast, Mordenti et al.25 reported that a full-length antibody could not cross the ILM and penetrate the retina, whereas the Fab fragment used in their study readily penetrated all layers of the retina. However, the two used species cannot be compared as easily, because the full-length antibody was hereceptin, which is directed against a human epidermal growth factor receptor (EGFR), whereas the Fab fragment was directed against VEGF. Recently, it has been reported that the EGFR is present in many retinal neurons, such as ganglion, amacrine, and horizontal cells in mice, rats, and humans.26 Given the widespread presence of its antigen in the inner retina, it would not be surprising if hereceptin did not penetrate very deeply because of specific binding, similar to the findings of Kamei et al.20 In contrast, the interpretation of the autoradiographic images (Fig. 1 in Mordenti et al.25) is questionable because of their irregular choice of the retinal sections in their Figures 1A to 1F. Although visibly slower, hereceptin seemed to penetrate the retina in the end, as indicated in Figure 1C in Mordenti et al.25

Similarly as in two studies23,24 the lgG protein bevacizumab also seems to penetrate the retina readily after its intravitreal injection. We were able to show this recently in mice (Heiduschka et al., manuscript in revision) and rabbits (Schaerermeyer et al. IOVS 2006;47: ARVO E-Abstract 4169). Shahar et al.27 also showed in a study performed in rabbits that bevacizumab is capable of penetrating the retina.

To summarize, we showed penetration of the retina by intravitreally administered bevacizumab in its commercial formulation. Although the needed therapeutic concentration of bevacizumab within the retina is not known, the amount of penetrating bevacizumab appears to be sufficient to elicit the therapeutic effects achieved in patients. Bevacizumab IR can be found in several cell types 14 days after its injection, most prominent of them rod PR and endothelial cells of blood vessels. The relationship between active transport of the injected protein by cells yet to be determined in detail and passive diffusion (probably promoted by the detergent Tween 20 in the commercial formulation) remains to be elucidated. Moreover, a detailed ultrastructural inspection of bevacizumab-injected retinas is currently ongoing (Peters et al., manuscript in press).

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References


