

Effects of Bevacizumab (Avastin) on Retinal Cells in Organotypic Culture

Stefanie Kaempf, Sandra Johnen, Anna Katharina Salz, Andreas Weinberger, Peter Walter, and Gabriele Thumann

PURPOSE. Repetitive intravitreal injections of bevacizumab are a successful treatment option for exudative age-related macular degeneration (AMD). The aim of this study was to evaluate the toxicity of bevacizumab in the adult mammalian neurosensory retina in culture.

METHODS. Adult porcine neurosensory retinas were cultured adjoined to the retinal pigment epithelium-choroid layer (retina-RPE-choroid complex) in static culture for 3 days, whereas neural retinas alone were cultured in a perfusion chamber for 3 days. Bevacizumab was added to the culture and perfusion medium at three concentrations (0.25 mg/mL [$n = 6$], 0.5 mg/mL [$n = 6$], and 1.25 mg/mL [$n = 6$]). Retina-RPE-choroid complex and neural retinas alone cultured without bevacizumab were used as controls. After 3 days in culture, the neural retinas alone and the retina-RPE-choroid complexes were analyzed histologically and immunohistochemically for the expression of glial fibrillary acidic protein (GFAP), vimentin, glutamine synthetase, rhodopsin, smooth muscle actin (SMA), and apoptosis.

RESULTS. No toxic effects on ganglion or photoreceptor cells were observed at any concentration of bevacizumab. The expression of GFAP and vimentin was slightly increased in Müller cells, whereas glutamine synthetase and rhodopsin were unaffected by bevacizumab. However, significantly enhanced SMA expression in retina blood vessels was observed in retinas cultured in the presence of bevacizumab.

CONCLUSIONS. Bevacizumab was well tolerated by ganglion and photoreceptor cells even at concentrations fivefold higher than those used clinically. The increased expression of SMA is an indication of the loss of functional VEGF modulating smooth muscle cells in mature vessels. (*Invest Ophthalmol Vis Sci* 2008;49:3164–3171) DOI:10.1167/iovs.07-1265

Treatment options for exudative age-related macular degeneration (AMD) associated with choroidal neovascularization have included laser treatment, membrane removal alone, and membrane removal coupled with macular translocation or pigment cell transplantation; however, none of these treatment modalities have resulted in significant visual improvement. Because substantial evidence has indicated that vascular

endothelial growth factor (VEGF)-A is a major mediator of angiogenesis and vascular leakage in exudative AMD,^{1–4} the inhibition of VEGF activity has been a central theme in many therapies under investigation. In fact, the inhibition of VEGF in animal models showed significant inhibition of retinal and choroidal neovascularization.^{5,6} A number of VEGF inhibitors have been developed for clinical use, including a VEGF-neutralizing oligonucleotide aptamer (pegaptanib [Macugen; Pfizer, New York, NY]),⁷ a humanized anti-VEGF monoclonal antibody fragment (ranibizumab),⁸ and VEGF receptor analog (sFlt-1).⁹

Pegaptanib, a synthetic pegylated oligonucleotide that binds only to one extracellular VEGF-165 isoform preventing its binding to the receptor, was the first anti-VEGF treatment approved for AMD patients.⁷ The results of intraocular injections of pegaptanib, however, have not been satisfactory.

Recently, ranibizumab (Lucentis; Genentech, San Francisco, CA), a humanized Fab fragment derived from the parent monoclonal bevacizumab, received United States Food and Drug Administration approval for intraocular use to treat AMD. It has undergone extensive clinical trials, and reports indicate substantially better outcomes in patients treated with intravitreal ranibizumab than conventional treatments in patients with exudative AMD. Recent clinical trials have shown that the inhibition of VEGF by repetitive injections of ranibizumab results in a median gain in visual acuity of 2 lines in the ETDRS chart in patients with exudative AMD.^{10–12} For optimal results, repetitive injections (usually monthly) of ranibizumab are necessary. Given that ranibizumab is very expensive (\$1500–\$2000 per 0.5-mg injection), a number of ophthalmologists have begun to experiment with intravitreal injections of 1.25 to 2.5 mg of the parent molecule bevacizumab (Avastin; Genentech) even though it is not approved for such use and is formulated for intravenous infusion but not intraocular injection.

Nonetheless, investigators have reported remarkable results in arresting the development of choroidal neovascularization in AMD and in proliferative diabetic retinopathy, neovascular glaucoma, diabetic macular edema, and macular edema secondary to retinal vein occlusions without any significant side effects.^{12–20}

In the United States, bevacizumab was approved for the treatment of metastatic cancer of the colon and rectum,²¹ and, although the price of bevacizumab as part of a chemotherapy regimen is approximately \$600 for a 4-mL vial, the 4-mL vial contains 100 mg. The physician or pharmacy can prepare small aliquots in syringes for intraocular injection at a cost to the physician of less than \$20. On a molar basis, the typical dose of 1.25 mg (0.05 mL) bevacizumab used for intravitreal injection is similar to the approved dose of ranibizumab.

Contrary to the extensive intraocular safety studies conducted for the approval of ranibizumab, few studies have been conducted on the intraocular safety of bevacizumab. In vitro studies have been reported on the effect of bevacizumab on ocular cells in culture, such as human retinal pigment epithelium (RPE), optic nerve head astrocytes, human corneal cells, rat retinal ganglion cells (RGC5), and pig choroidal endothelial cells.^{22–24} The safety of bevacizumab has been tested in vivo in

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rabbits²⁵⁻²⁷ and monkeys.^{28,29} A small, prospective, phase 1, dose-escalation study has confirmed good tolerance of bevacizumab after intravitreal injection in AMD patients.¹²

In this study we have analyzed the distribution and characterized the effects of bevacizumab in an organ culture system of adult mammalian retinal tissue. Our results show that bevacizumab does not alter significantly the expression of cell-specific proteins except for the large increase in smooth muscle actin in retinal vessels, suggesting that the inhibition of VEGF may alter vessel permeability in the retina.

MATERIALS AND METHODS

Tissue Isolation

Porcine eyes were obtained from a local slaughterhouse within 1 hour of kill and were transported to the laboratory on ice. The eyes were washed once for 3 minutes with 70% ethanol; this was followed by ice-cold sterile phosphate-buffered saline (PBS) containing 100 U/mL penicillin and 0.1 mg/mL streptomycin. The anterior segment was cut approximately 3.5 mm posterior to the limbus, and the posterior segment was flattened by a radial incision of the sclera. The globe was turned over, and the vitreous was carefully removed using a sterilized cotton swab. The neurosensory retina, which becomes detached during the removal of the vitreous, was washed in situ with PBS, with the inner limiting membrane (ILM) facing downward on a sterile ceramic tile, and the connection to the optic nerve head was cut using scissors. The neural retina was separated to avoid damaging it during separation of the RPE-choroid from the sclera. Using a cell scraper, the RPE-choroid complex was separated from the underlying sclera and placed on a nitrocellulose membrane (Whatman Schleicher & Schuell, Dassel, Germany; pore size, 0.45 μm) with the choroid facing downward. Unless otherwise noted, all chemicals, tissue culture media, and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany).

Tissue Culture

Immediately after isolation, the neural retina and the RPE-choroid tissue were recombined, restoring the original in vivo orientation. Then pieces of 10 \times 10 mm were cut using a surgical blade. The tissue was placed on a sterile, perforated, high-grade stainless steel grid support bench placed in a six-well culture plate, and the wells were filled with minimal essential medium (MEM) culture up to a level just below the upper vitreous surface of the explant to accommodate the high oxygen demand of the neural retina. The culture medium con-

sisted of MEM (Gibco Brl, Eggenstein, Germany), supplemented with 2.8 mM L-glutamine (Gibco), 10% newborn calf serum (Gibco), 673 U/mL penicillin, and 673 $\mu\text{g}/\text{mL}$ streptomycin. The culture medium was changed every 24 hours. Bevacizumab was added to the culture medium at three concentrations: 0.25 mg/mL ($n = 6$), 0.5 mg/mL ($n = 6$), and 1.25 mg/mL ($n = 6$).

Perfusion Retinal Culture

Neural retina specimens were placed on nitrocellulose membranes and transferred to a sterile cell carrier (Minusheet; Vertriebs GmbH, Bad Abbach, Germany). Six cell carriers were placed in a perfusion culture chamber (Minucells and Minutissue; Vertriebs GmbH) and perfused with 1 mL/h complete medium (MEM supplemented with 2.8 mM L-glutamine, 10% newborn calf serum, 673 U/mL penicillin, and 673 $\mu\text{g}/\text{mL}$ streptomycin). The effect of bevacizumab was examined by adding it to the perfusion culture medium at 0.25 mg/mL ($n = 6$), 0.5 mg/mL ($n = 6$), and 1.25 mg/mL ($n = 6$).

Immunohistochemistry

After 3 days of culture, the retina-RPE-choroid and neural retina specimen were fixed for 24 hours in 4% paraformaldehyde in PBS (vol/vol) at room temperature. After dehydration in an ascending series of ethanol (70%, 96%, 100%; vol/vol) the tissue was transferred to xylol and embedded in paraffin according to standard protocols. Three-micrometer-thick serial sections were mounted on poly-L-lysine-coated slides and dried at 37°C for 24 hours. Before processing for immunohistochemistry, sections were deparaffinized in xylene and hydrated in a graded series of ethanol (100%, 96%, 70%; vol/vol and water), and sections from all cultured tissues were stained with hematoxylin-eosin (H&E) and analyzed by light microscopy (Zeiss Axioskop, Oberkochen, Germany) for morphologic damage; only histomorphologically intact tissues were processed for immunohistochemistry. For immunohistochemistry, deparaffinized sections were transferred into citrate buffer (0.1 M, pH 6) and irradiated by microwave for 15 minutes at 500 W to increase permeabilization and unmask antigens. After stepwise exchange of citrate buffer by PBS and cooling, sections were washed with PBS and blocked with 20% normal goat serum (NGS) and 2% BSA in PBS (vol/vol) for 1 hour. Sections were incubated at room temperature with the appropriate primary antibody diluted in 10% NGS and 2% BSA in PBS. After 3 hours of incubation with the primary antibodies, the sections were washed three times, 5 minutes each time, with PBS, and the presence of the primary antibody was detected by incubating the sections in the dark for 3 hours with the secondary

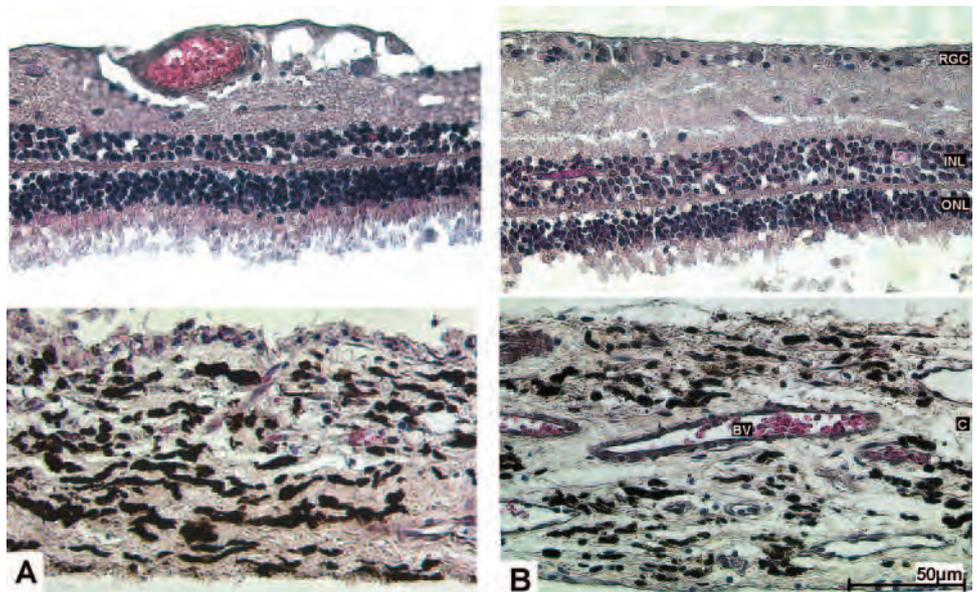


FIGURE 1. Light micrographs of retina and RPE-choroid cultured in medium without (A) and with (B) the addition of 1.25 mg/mL bevacizumab. All retinal layers were well preserved after static culture, and no differences were evident between treated and untreated retinas. Similar histologic results were observed in perfusion cultures (not shown). Separation of retina and RPE-choroid was a result of paraffin embedding. C, choroid; BV, blood vessel.

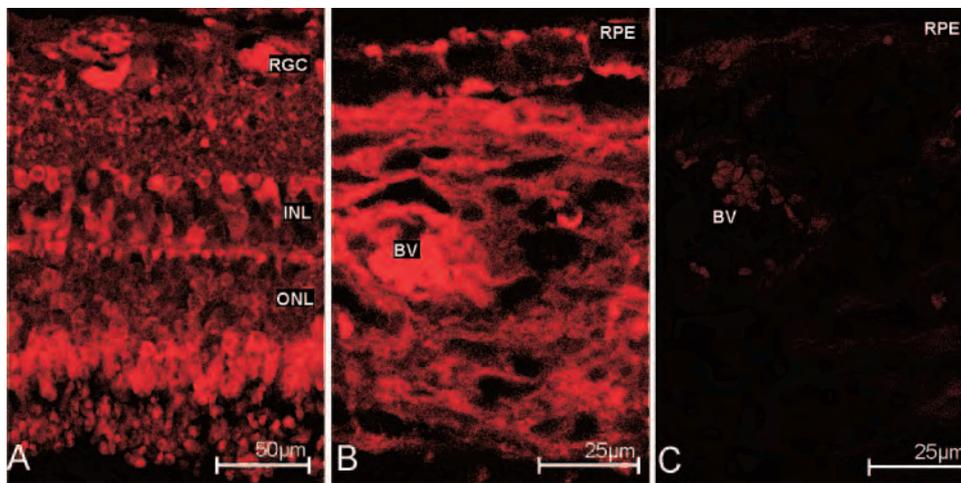


FIGURE 2. Immunohistochemical localization of bevacizumab in retina-RPE-choroid cultures. Bevacizumab (1.25 mg/mL) is localized throughout all retinal layers, retinal vessels (A), choroid, and RPE (B), as demonstrated by red fluorescence, whereas in control culture without bevacizumab no relevant red fluorescence by the Cy3-antibody is visible (C). BV, blood vessel.

antibody goat anti-mouse IgG (H+L) or goat anti-rabbit IgG (H+L) (Alexa Fluor 488; Molecular Probes, Göttingen, Germany) at a 1:500 dilution in 10% NGS and 2% BSA in PBS. To control for nonspecific binding by the secondary antibody, the primary antibody was omitted. After three more washes, 5 minutes each time, with PBS, the sections were incubated for 5 minutes with DAPI (350 μ g DAPI/mL in 50% ethanol, 50% PBS; Merck, Darmstadt, Germany) for nuclear staining. After the DAPI solution was removed, the slides were covered with a few drops of glycine/PBS (9:1; vol/vol) and were analyzed by fluorescence microscopy.

Sections were analyzed for expression of the following proteins: glial fibrillary acidic protein (GFAP) as an indicator of astrocyte and glial cells, glutamine synthetase (GS) as an indicator of Müller cells, vimentin as an indicator of cytoskeleton and cell shape, rhodopsin as an indicator of photoreceptors, and smooth muscle actin (SMA) as an indicator of smooth muscle and pericytes of blood vessels. The following primary antibodies were used: anti-GFAP, mouse monoclonal antibody, clone G-A-5, (1:100; catalog number G-3893; Sigma, Munich, Germany); anti-GS, rabbit polyclonal antibody (1:300; catalog number G 2781; Sigma); anti-vimentin, mouse monoclonal antibody, clone V9, (1:100; catalog number V-6630; Sigma); anti-rhodopsin, mouse monoclonal antibody, clone 1D4 (1:500; catalog number R-5403; Sigma); anti-SMA, mouse monoclonal antibody, clone 1A4 (1:100; catalog number M0851; Dako, Hamburg, Germany), and anti-VEGF, chicken polyclonal (1:50; catalog number ab14078; Abcam, Cambridge, UK). Sections were analyzed for localization of antigens using fluorescence microscopy and were photographed under a microscope (Polyvar; Leica, Bensheim, Germany).

Bevacizumab was localized by immunocytochemistry using Cy3-conjugated donkey anti-human IgG (H+L, 1:100; Jackson ImmunoResearch, Suffolk, UK) according to the procedure described. Sections

were observed and photographed using a confocal laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany).

In Situ Detection of Apoptosis by TUNEL Staining

Detection of apoptotic cells was performed using TUNEL staining according to the manufacturer's protocol (In situ Cell Death Detection kit, Fluorescein; Roche, Penzberg, Germany). Deparaffinized sections of cultured retinas were microwaved for 3 minutes at 360 W in citrate buffer (0.1 M, pH 6). After the stepwise exchange of hot citrate buffer with PBS at room temperature, sections were incubated in Proteinase K (20 μ g/mL in 10 mM Tris HCl, pH 7.5; Sigma) for 30 minutes, washed 3 times with PBS, and incubated in the TUNEL reaction mixture for 1 hour at 37°C in the dark. The TUNEL reaction mixture contained terminal deoxynucleotidyl transferase (TdT), which catalyzes the polymerization of fluorescein-labeled nucleotides to the 3'-OH DNA ends in a template-independent manner (TUNEL reaction). Negative control sections were incubated without TdT. Sections were counterstained with DAPI and analyzed using fluorescence microscopy.

The number of apoptotic cells is expressed as the ratio of DAPI-TUNEL double-labeled nuclei to the total number of nuclei stained with DAPI. Cells were counted in two fields in six micrographs (one representative micrograph per culture; $n = 6$) at a magnification of 40 \times . All values are presented as mean \pm SD ($n = 12$); data were analyzed for statistical significance by the Mann-Whitney rank sum test. $P < 0.05$ was considered statistically significant.

Western Blot Analysis

To evaluate whether bevacizumab exhibits species cross-reactivity and binds to porcine VEGF, porcine and human retinal tissues were homogenized in SDS lysis buffer (0.5 M Tris-HCl, pH 6.8, 2% SDS [wt/vol],

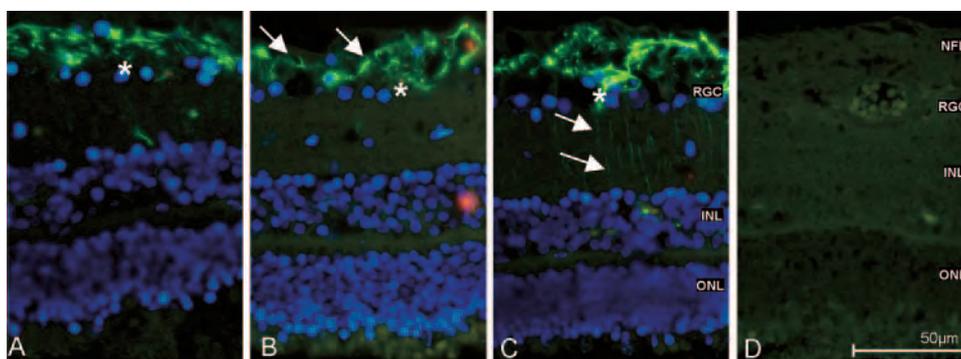
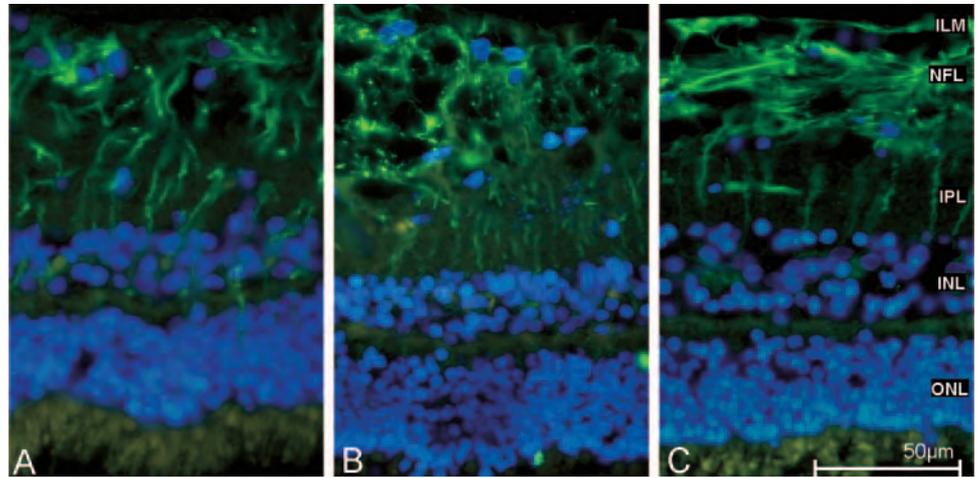


FIGURE 3. Immunohistochemical visualization of GFAP in neural retina cultures. In retinas cultured without bevacizumab (A), GFAP is detected immunohistochemically in the astrocytes of the NFL, in the astrocytes close to blood vessels, and in outer radial processes of Müller cells until the outer plexiform layer (OPL). After 3 days in perfusion culture of retinas in the presence of 0.25 mg/mL bevacizumab (B), no changes in the distribution or intensity of GFAP were observed (arrows). In retina culture with 1.25 mg/mL bevacizumab, a slight increase of GFAP in the apical Müller cell processes in the IPL is evident (arrows) (C). Nuclear stain with DAPI shows a stable number of nuclei in the RGC layer, most likely ganglion cells in all cultures (asterisk). In the control sections incubated only with secondary antibody (Alexa Fluor 488 goat anti-mouse IgG (H+L)), no green fluorescence is evident (D). NFL, nerve fiber layer.

sections were analyzed for expression of the following proteins: glial fibrillary acidic protein (GFAP) as an indicator of astrocyte and glial cells, glutamine synthetase (GS) as an indicator of Müller cells, vimentin as an indicator of cytoskeleton and cell shape, rhodopsin as an indicator of photoreceptors, and smooth muscle actin (SMA) as an indicator of smooth muscle and pericytes of blood vessels. The following primary antibodies were used: anti-GFAP, mouse monoclonal antibody, clone G-A-5, (1:100; catalog number G-3893; Sigma, Munich, Germany); anti-GS, rabbit polyclonal antibody (1:300; catalog number G 2781; Sigma); anti-vimentin, mouse monoclonal antibody, clone V9, (1:100; catalog number V-6630; Sigma); anti-rhodopsin, mouse monoclonal antibody, clone 1D4 (1:500; catalog number R-5403; Sigma); anti-SMA, mouse monoclonal antibody, clone 1A4 (1:100; catalog number M0851; Dako, Hamburg, Germany), and anti-VEGF, chicken polyclonal (1:50; catalog number ab14078; Abcam, Cambridge, UK). Sections were analyzed for localization of antigens using fluorescence microscopy and were photographed under a microscope (Polyvar; Leica, Bensheim, Germany).

FIGURE 4. Immunohistochemical visualization of vimentin in neural retina cultures. Müller cells in retinas cultured in the presence of 0.25 mg/mL bevacizumab (**B**) show no alteration, but in the presence of 1.25 mg/mL bevacizumab (**C**), they show a moderate increase of vimentin expression compared with control perfusion culture without bevacizumab (**A**). Müller cells are stained from their vitreal end feet (ILM, NFL) until the apical processes in the IPL and somata in the INL. Nuclei stained with DAPI. NFL, nerve fiber layer; IPL, inner plexiform layer.



and 10% glycerol [vol/vol]) containing a protease inhibitor cocktail (104 mM AEBSEF, 0.08 mM aprotinin, 2.2 mM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A, 1.4 mM E-64; Sigma). The homogenates were centrifuged at 10,000g for 15 minutes at 4°C. An aliquot of the supernatant containing 25 µg protein from the porcine and human retinal tissue was incubated with 0.25 mg/mL bevacizumab for 10 minutes at room temperature, electrophoresed on a 10% SDS polyacrylamide gel under reducing conditions, and electrotransferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was washed with PBS-Tween-20 for 20 minutes at room temperature, blocked with 5% nonfat milk in PBS containing 0.05% Tween-20 (TBS-Tween) for 30 minutes, and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated goat anti-human IgG (AbD Serotec, Oxford, UK; 0500-0099; 1:5000). After washing the membrane with TBS-Tween-20, protein was visualized with a chemiluminescence-based procedure using the detection kit (ECL Plus; Amersham) according to the manufacturer's protocol.

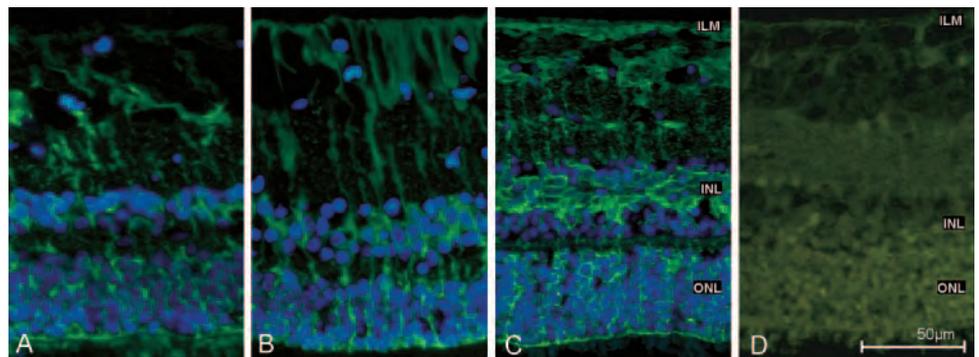
RESULTS

Histomorphologic observation of H&E-stained static cultures of retina-RPE-choroid and of neural retinas in perfusion culture showed a well-preserved nuclear layer, well-preserved ganglion cells and photoreceptors, and a normal choroid after exposure to bevacizumab (Fig. 1). As labeled by anti-human conjugated Cy3-antibody, bevacizumab was localized not only in all retinal layers in static and in perfusion cultures (Fig. 2A) but also in the choroid (Fig. 2B). Although most of the bevacizumab labeling appeared to localize in the extracellular spaces of retina and choroid (Figs. 2A, 2B), some nuclei of the retinal ganglion cells (RGCs), most likely RGCs and cell bodies of the inner nuclear layer (INL; Fig. 2A) and RPE (Fig. 2B), appear to have internalized the antibody. Control cultures in the absence

of bevacizumab did not show any nonspecific binding of the secondary antibody (Cy3-conjugated donkey anti-human IgG; Fig. 2C). The very slightly elevated levels of GFAP (Figs. 3B, 3C) and vimentin (Figs. 4B, 4C) and the similar levels of GS expression (Figs. 5B, 5C) in control (Figs. 3A, 4A, 5A) and bevacizumab-treated retinas in neural retina perfusion culture and static retina-RPE-choroid culture (not shown) indicate that bevacizumab did not cause significant glial hypertrophy at any of the concentrations used. Control sections incubated only with the secondary antibodies goat anti-mouse IgG or goat anti-rabbit IgG (Alexa Fluor 488; Mobitec) did not show any fluorescence (Figs. 3D, 5D). Photoreceptors were also well preserved, as evidenced by similar rhodopsin expression in treated (Figs. 6B, 6C) and control retinas (Fig. 6A) in neural retina cultures and in retina-RPE-choroid cultures (not shown). Treatment with bevacizumab induced a significant increase in SMA expression in the smooth muscle cells and pericytes of mature retinal vessels (Figs. 7A-D) in retinal perfusion cultures and in retina-RPE-choroid cultures.

Morphologic signs of retinal damage, such as thinning of retinal layers, degeneration, and loss of photoreceptor outer segments or loss of nuclei in the retinal ganglion cell layer or nuclear layers, which indicate neuronal cell death, were not observed in any retinal culture with bevacizumab or control retina cultures. In retina-RPE-choroid cultures without bevacizumab, apoptotic cells were seen in the ganglion cell layer ($25.2\% \pm 2.5\%$), INL ($16.0\% \pm 3.5\%$), ONL ($9.9\% \pm 2.0\%$), and RPE-choroid ($21.8\% \pm 3.1\%$) (Figs. 8A, 8C, 9). Similar levels of apoptotic cells were observed in bevacizumab-treated cultures; in cultures of retina-RPE-choroid in the presence of 1.25 mg/mL bevacizumab, no increase in apoptotic cells was observed by TUNEL labeling (Figs. 8A-D) or quantification (Fig. 9). In retina-RPE-choroid cultures in the presence of 1.25

FIGURE 5. Immunohistochemical visualization of glutamine synthetase in neural retina cultures. Retinas in perfusion culture in the presence of bevacizumab do not show any alteration of glutamine synthetase expression throughout the entire retina. (**A**) Control, without bevacizumab. (**B**) 0.25 mg/mL bevacizumab. (**C**) 1.25 mg/mL bevacizumab. Nuclear bodies are stained with DAPI. No fluorescence is apparent in control section in the absence of primary antibody (**D**). BV, blood vessel.



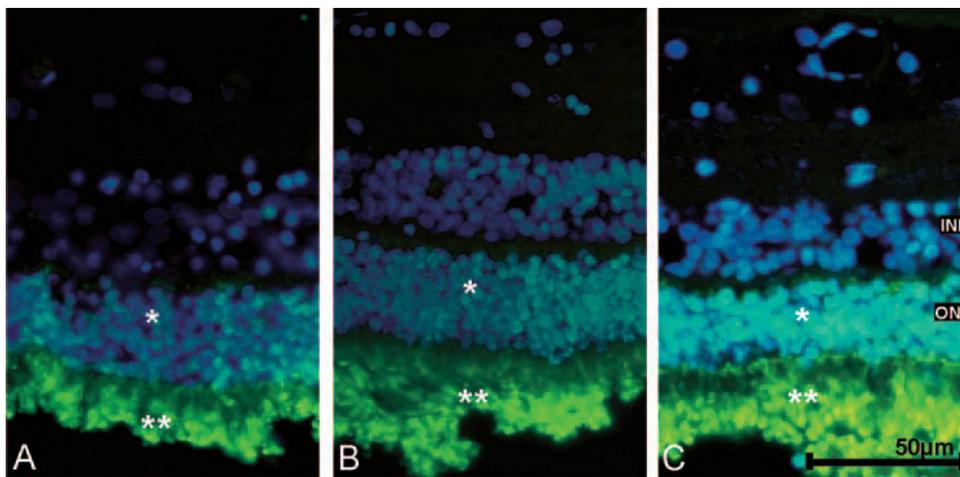


FIGURE 6. Immunohistochemical visualization of rhodopsin in neural retina cultures. Photoreceptor cell inner (single asterisks) and outer (double asterisks) segments were well maintained in untreated retina cultures (A) and in bevacizumab-treated retinas (0.25 mg/mL bevacizumab (B); 1.25 mg/mL bevacizumab (C)) after 3 days of perfusion culture, as shown by comparable stain intensity with anti-rhodopsin antibody. Nuclear bodies were stained with DAPI.

mg/mL bevacizumab, $27.8\% \pm 3.6\%$ of ganglion cell layer (GCL) nuclei, $18.9\% \pm 3.4\%$ of INL nuclei, $9.2\% \pm 1.5\%$ of ONL cell bodies, and $21.1\% \pm 2.1\%$ RPE-choroid were TUNEL positive.

Western blot analysis of human and porcine retinal tissue (Fig. 10) shows that bevacizumab cross-reacts with the porcine VEGF165 isoform (23 kDa).

DISCUSSION

Bevacizumab (Avastin; Genentech) is a full-length humanized murine monoclonal antibody against the VEGF molecule, and it binds to all isoforms of VEGF (VEGF₁₁₀, VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₃, VEGF₁₈₉, VEGF₂₀₆). Compared with ranibizumab, bevacizumab is three times larger and shows a significantly lower binding affinity to VEGF.³⁰ The half-life of bevacizumab is 40 times longer than the half-life of ranibizumab^{31,32}; however, intravitreally the half-life appears to be approximately 3 days for both antibodies.^{27,33,34} Even though intravitreal injection uses minute amounts of the drug, the full-size antibody, bevacizumab, has more potential to cause inflammatory and immune reactions over time.

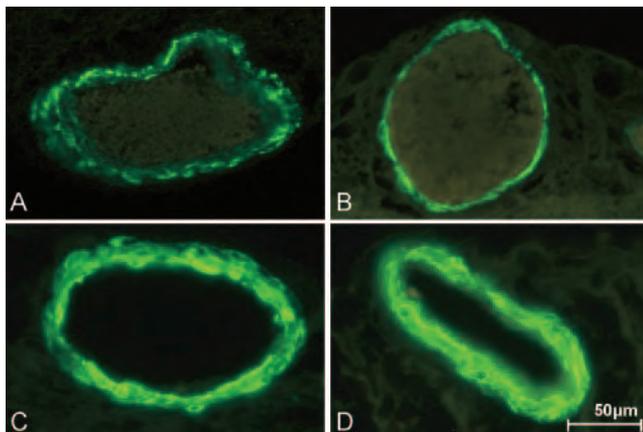


FIGURE 7. Immunohistochemical visualization of smooth muscle actin (SMA) in neural retina cultures. A section of in situ retinal tissue shows SMA expression in vascular smooth muscle cells (A). A similar pattern and a similar intensity of SMA expression are observed in retinal tissue cultured in control medium without the addition of bevacizumab (B). The addition of 0.25 mg/mL bevacizumab (C) and 1.25 mg/mL bevacizumab (D) to the culture medium resulted in a significant increase in the expression of SMA in smooth muscle cells of mature retinal vessels.

To avoid possibly severe systemic side effects, treatment of ocular disease is usually limited to intraocular administration, in which case retinal toxicity is the primary concern. Information on the specific effects of bevacizumab on individual retinal cells is sparse.

Manzano et al.²⁵ did not observe significant changes in the electroretinograms of albino rabbits or signs of retinal toxicity after intravitreal administration of bevacizumab at concentrations ranging from 0.025 mg/mL to 2.5 mg/mL. Shahar et al.²⁷ injected bevacizumab into 10 albino rabbits using a single therapeutic intravitreal concentration of 1.25 mg/mL. Electroretinography and visual-evoked potentials showed normal patterns, indicating no toxicity during 4 weeks of follow-up.

With the use of immunohistochemistry, these authors showed that bevacizumab penetrated the full retina within 24 hours of injection.²⁷ By 7 days, bevacizumab labeling was primarily observed extracellularly, similar to our results. In monkeys, systemic blood levels of bevacizumab are recorded as early as 4 hours after intravitreal injection (Cousins SW, et al. *IOVS* 2007;48:ARVO E-Abstract 22). After intravitreal injection, bevacizumab is detected in all layers of the retina by immunohistochemistry, specifically in the ILM, the ganglion cells, the INL, the inner and outer segments of photoreceptors, and, in some cases, in Müller cells (Cousins SW, et al. *IOVS* 2007;48:ARVO E-Abstract 22).²⁷⁻²⁹

Luke et al.³⁵ exposed freshly isolated bovine retinas to bevacizumab for 45 minutes at concentrations ranging from 0.08 mg/mL to 0.8 mg/mL, followed by a 60-minute perfusion with medium without bevacizumab. Simultaneous recording of ERG did not show any reduction of the a- or b-wave amplitude at any concentration, indicating that the retinal neurons preserved function at least for the 60 minutes of perfusion.

Given that a number of investigators have reported improved visual acuity in AMD patients after bevacizumab treatment,³⁶ it is essential to understand whether bevacizumab has any adverse effects on the retina. Our approach to this question has been to study the effects of bevacizumab on the expression of specific proteins and of neural cell survival in retina-RPE-choroid cocultures and in isolated perfused neural retinas. The results of our studies have shown that in static and perfused cultures, the apoptosis of neuroretinal cells did not increase and the expression of proteins specific for neural retinal cells was not altered significantly, suggesting that bevacizumab does not have any toxic effects on the neural retina. Bevacizumab, an antibody against human VEGF, cannot bind to murine VEGF because of structural differences between the human and the murine binding site.³⁷ Here we have shown that bevacizumab cross-reacts with porcine VEGF, as demonstrated by the specific binding to porcine VEGF165 by Western

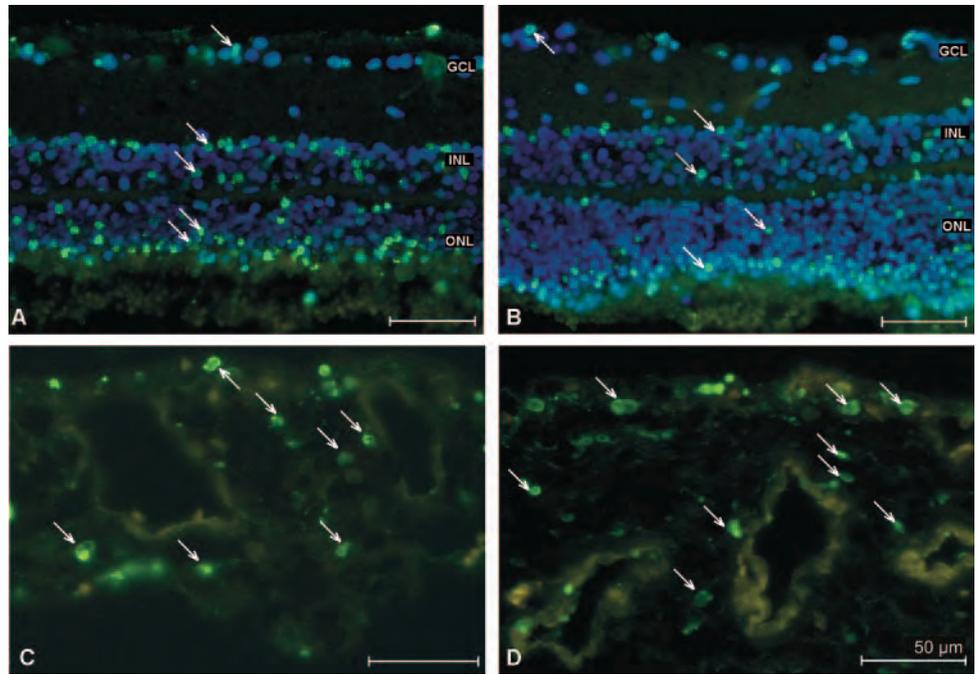


FIGURE 8. Detection of apoptotic cells by TUNEL staining. Fluorescence photomicrographs of retina-RPE-choroid cultures in the absence of bevacizumab (**A, C**) and in the presence of 1.25 mg/mL bevacizumab (**B, D**) processed with TUNEL technique (green) and counterstained with DAPI (blue) show a similar number of apoptotic cells (arrows) in the GCL, INL, and ONL (**A, B**) and in the RPE and choroid (**C, D**).

blots, validating the significance of our results. In contrast to the slightly increased expression of GFAP and vimentin, retinal cells showed normal expression of GS and rhodopsin. A moderate reaction of Müller cells, as indicated by the upregulation of GFAP and vimentin, cannot be excluded. Most important, the expression of SMA in retinal blood vessel cells was upregulated significantly by bevacizumab. The upregulation of SMA in retinal vessels has not been previously reported, and careful evaluation of the effects of increased SMA in retinal function is essential. In addition, it will be important to determine whether ranibizumab also upregulates SMA in retinal vessels.

Smooth muscle cells and pericytes ensheath the endothelial tube of retinal arteries, arterioles, and veins.³⁸ Smooth muscle α -actin is necessary for vessel stability and a functional blood retinal barrier.^{39,40} VEGF not only induces the produc-

tion of nitric oxide and prostacyclin, it inhibits smooth muscle cell hyperplasia and proliferation.^{41,42} Inhibition of VEGF could attenuate its antiproliferative and antithrombotic function and promote smooth muscle cell hyperplasia and proliferation.⁴⁰ Our findings could explain the known cardiovascular side effects of VEGF inhibition, namely stroke and myocardial infarction.⁴³ Similarly, an increase in SMA could explain the increased thromboembolic events that have been reported in phase 3 clinical trials using systemic bevacizumab⁴⁴ and the thrombocyte activation and choroidal thromboembolic plaques that have been observed after intravitreal injection of bevacizumab in monkeys.²⁹

We hypothesize that the rapid upregulation of SMA after the inhibition of VEGF is responsible for the rapid decrease in exudation and, therefore, retinal vascular permeability seen clinically in AMD patients after anti-VEGF treatment.

In conclusion, our study demonstrates that bevacizumab is well tolerated by neuroretinal cells; however, possible side

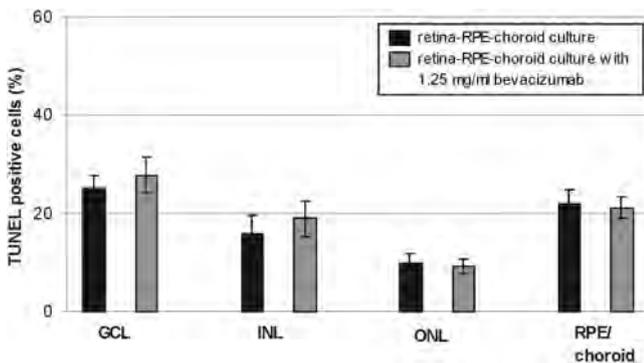


FIGURE 9. Quantitation of apoptotic cells. The number of apoptotic, TUNEL-positive cells is expressed as the ratio of DAPI-TUNEL double-labeled nuclei to the total number of DAPI-stained nuclei. In cultures of retina-RPE-choroid in the absence of bevacizumab, the number of apoptotic cells was 25.2% ± 2.5% in the GCL, 16.0% ± 3.5% in the INL, 9.9% ± 2.0% in the ONL, and 21.8% ± 3.1% in the RPE-choroid. In retina-RPE-choroid cultured in the presence of bevacizumab, the percentages of apoptotic cells were similar to those in cultures in the absence of bevacizumab: 27.8% ± 3.6% in the GCL, 18.9% ± 3.4% in the INL, 9.2% ± 1.5% in the ONL, and 21.1% ± 2.1% in the RPE-choroid (mean ± SD; $n = 12$; Mann-Whitney rank sum test; $P < 0.05$).



FIGURE 10. Western blot of porcine and human retinal homogenates using bevacizumab as primary antibody shows that bevacizumab reacts with a protein of approximately 25 kDa in the retinal homogenates of porcine (lane 1) and human (lane 2) retinal tissue. Because bevacizumab reacts with a number of VEGF isoforms, the 25-kDa protein represents VEGF165. Lane 3 shows the protein marker migration.

effects on mature vessels must be considered and may explain the higher risk for cardiovascular events in systemic anti-VEGF treatments. In the eye, the upregulation of SMA after the inhibition of VEGF by bevacizumab, and possibly other VEGF inhibitors, will require careful study because, in the long term, increased SMA could disrupt the normal function of the retinal vasculature—namely, supplying essential nutrients and eliminating waste—and could lead to neural retina damage.

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