Comparative Evaluation of Apoptotic Activity in Photoreceptor Cells after Intravitreal Injection of Bevacizumab and Pegaptanib Sodium in Rabbits

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PURPOSE. To evaluate quantitatively the apoptotic activity after intravitreal injections of pegaptanib sodium and bevacizumab in the rabbit retina.

METHODS. Different doses of bevacizumab (0.25, 0.625, 1.25, and 2.5 mg) and pegaptanib sodium (0.15, 0.3, and 0.6 mg) were injected intravitreally in 48 rabbits. The eyes were enucleated at different times for early studies at day 14 and for late studies at 3 months after a single injection or at 3 months, with 1 injection in each of the 3 months (day 90). The time course and dose-response of photoreceptor cells in the rabbit retina after intravitreal injection of bevacizumab or pegaptanib sodium were examined by histologic analysis with hematoxylin and eosin (H&E) staining, caspase-3 and -9 immunostaining, and in situ terminal-deoxynucleotidyl transferase-mediated biotin-deoxyuridine triphosphate nick-end labeling (TUNEL) of DNA fragments of paraffin-embedded sections.

RESULTS. No sign of retinal toxicity was seen in H&E stained histologic sections of eyes that had received bevacizumab or pegaptanib sodium. Nuclear DNA fragmentation in the outer retinal layers shown by the TUNEL method was evident in the high-dose groups (55.3% with 1.25 mg and 64.5% with 2.5 mg bevacizumab, and 48.5% with 0.6 mg pegaptanib sodium) at 14 days and also in the clinical dose groups (49.8% with three injections [1 each month] of 0.625 mg bevacizumab and 44.3% with 0.15 mg pegaptanib sodium) at 90 days. The ratios of TUNEL-positive cells in physiologic saline and the sham-control groups were 32.3% and 21%, respectively.

CONCLUSIONS. Intravitreal injection of bevacizumab and pegaptanib sodium caused a significant increase in apoptotic activity in rabbit photoreceptor cells. However, although bevacizumab caused increased apoptotic activity at higher doses, similar dose-dependent adverse effects were not evident for pegaptanib sodium. (Invest Ophthalmol Vis Sci. 2009;50:3438–3446) DOI:10.1167/iovs.08-2871

Angiogenesis has an important role in choroidoretinal vascular diseases such as proliferative diabetic retinopathy, age-related macular disease (ARMD), and retinopathy of prematurity. The effects of VEGF antagonists on ocular neovascularization have raised new hope for the treatment of proliferative vascular diseases.3,4

Bevacizumab is an anti-VEGF humanized monoclonal antibody that binds to all biological active isoforms of VEGF. It is the first antiangiogenic agent to be approved by the FDA for the treatment of metastatic colorectal cancer in conjunction with chemotherapy. Although bevacizumab cannot theoretically penetrate all retinal layers efficiently because of its large size, it penetrates to deeper retinal layers, and clinically promising results have been reported after intravitreal application in the treatment of subretinal neovascularization.5–8 Despite ongoing clinical trials with low-dose intravitreal bevacizumab application in various neovascular ocular disorders, its ocular safety is still an issue for research. Several preclinical experimental toxicity studies have reported the histopathologic effects of bevacizumab on retinal cells, retinal neovascular membranes, and capillaries in paraffin-embedded sections6–9 or organotypic culture10–12 as well as in ultrastructural evaluations.13,15,14

Pegaptanib sodium, an anti-VEGF RNA aptamer specific to the VEGF165 isoform, has been used for the treatment of all types of neovascular ARMD.15–17 Clinical studies have shown that the inhibition of VEGF by intravitreal pegaptanib sodium results in the stabilization or improvement of vision in patients with neovascular ARMD; and, unlike bevacizumab, it has been produced for intraocular application15,16–20 However, there are limited preclinical studies associated with the intravitreal administration of pegaptanib sodium in vivo21 and in vitro.10,12

In our previous study, we investigated early and late-term retinal toxicity of bevacizumab by light and electron microscopic study in rabbit eyes and found ultrastructural changes in the mitochondria in the inner segments of the photoreceptors, suggesting increased apoptotic activity. The results of the immunohistochemical study with caspase-3, caspase-9, and bax/bcl-2 staining supported apoptotic activity in photoreceptor cells.13

In the present study, we investigated the quantitative evaluation of apoptotic activity, mainly by using the TUNEL (in situ terminal-deoxynucleotidyl transferase-mediated biotin-deoxyuridine triphosphate nick-end labeling of DNA fragments) method in rabbit retinal cells after application of intravitreal bevacizumab and also pegaptanib sodium with different dose schedules.

MATERIALS AND METHODS

Full-length humanized rhuMAB vascular endothelial growth factor (VEGF) antibody (bevacizumab, Avastin; Genentech/Roche, San Francisco, CA) and an RNA aptamer directed against VEGF-165 (pegaptanib sodium, Macugen; Pfizer Ltd., New York, NY), the VEGF isoform primarily responsible for pathologic ocular neovascularization and vascular permeability were used in the study.

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Animals

The clinical and experimental protocols were approved by the Animal Care and Use Committee of Uludag University and are in accordance with the guidelines set forth by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the institutional guidelines regarding animal experimentation in ophthalmic and vision research. The eyes used in the study were taken from adult male albino rabbits obtained from the Experimental Animal Breeding and Research Centre of Uludag University. The animals were fed standard laboratory food and allowed free access to water in an air-conditioned room with a 12-hour light–dark cycle.

Forty eight adult male rabbits weighing 1.8 to 2.5 kg each on the day before drug administration were assigned to 11 study groups (n = 4 in each group) and one control group (n = 2, 4 eyes) for sham control and one saline-injection control group (n = 2, 4 eyes; Table 1). By assuming rabbit vitreous volume as 2.5 mL and human vitreous as approximately 5 mL, the doses of 0.625 mg (25 µL, 25 mg/mL) bevacizumab and 0.15 mg (45 µL, 3.47 mg/mL) pegaptanib sodium were regarded as clinical doses because the doses of 1.25 and 0.3 mg, respectively, are used clinically in humans.22 Early (14 days) groups were assigned doses below (0.25 mg) and above (1.25 and 2.5 mg) the clinical doses for eyes receiving bevacizumab.

Groups 1 to 4 received 0.25, 0.625, 1.25 and 2.5 mg intravitreal single-dose bevacizumab, respectively, and groups 7 to 9 received 0.15, 0.3, and 0.6 mg intravitreal single-dose pegaptanib sodium, respectively. Groups 1 to 4 and 7 to 9 were labeled as early groups and scheduled to be killed on day 14. Right eyes were assigned as study eyes. Group 5 received a single 0.625-mg dose of intravitreal bevacizumab and group 6 received one injection of 0.625-mg intravitreal bevacizumab in three consecutive months. Group 10 received a 0.15-mg single dose pegaptanib sodium and group 11 received three monthly injections of 0.15 mg intravitreal pegaptanib sodium. Groups 5, 6, 10, and 11 were labeled as late groups and scheduled to be killed at the end of month 3. Group 12 (both eyes of two rabbits) received 0.05 mL physiologic saline (balanced salt solution, BSS Plus; Alcon, Fort Worth, TX) intravitreally for the saline control. Group 15 consisted of both eyes of two rabbits that did not receive any injection, but were exposed to the press of a needle tip for sham control. After the rabbits were killed, the study and control eyes were enucleated and preserved for light microscopic evaluation.

Procedure

Intravitreal injections were performed after a baseline ophthalmic examination with a handheld slit lamp biomicroscope (Kowa Ltd., Nagoya, Japan), determination of intraocular pressure (Tono-pen XL; Medtronic, North Jacksonville, FL), and fundoscopy by indirect ophthalmoscopy. Before all intravitreal injections, the rabbits were anesthetized by intramuscular injection (0.5 mL/kg body weight) of a mixture containing ketamine hydrochloride (25 mg/kg) and xylazine hydrochloride (2 mg/kg) solution.

The pupils were fully dilated with 2.5% phenylephrine hydrochloride and 1% tropicamide. After ocular surface anesthesia with a topical instillation of proparacaine (Alcain; Alcon, Fort Worth, TX) to reduce the animal’s discomfort, the eyes were washed with several drops of 5% povidone iodide. A 27-gauge standard disposable needle for bevacizumab injection or 27-gauge needle of prefilled syringe for pegaptanib sodium was introduced into the vitreous cavity 1.0 to 1.5 mm posterior to the superotemporal limbus, and the needle tip was directed into the midvitreous under direct visualization with external illumination of an indirect ophthalmoscope. One volume of the drug solution was then slowly administered according to the group allocation of the animal. The needle was held in place for 10 seconds before withdrawal, to prevent reflux from the entry site. The central retinal artery was observed by indirect ophthalmoscopy to ensure patency after each injection. Ofloxacin eye drops (0.3% Exocin; Allergan, Irvine, CA) were applied to the eyes of the experimental group animals, immediately after the procedure.

Toxicity Assessment

Clinical Observations. Study eyes were clinically examined, first before the injection, then 3 days after, and finally at the end of the study. At follow-up the pupils were dilated with tropicamide eye drops. According to a prospective protocol, the following parameters were recorded: corneal clarity, transparency, appearance of the lens and retina, conjunctival reaction, proteinaceous ray, and cells in the anterior and posterior segments of the eye.

At the baseline, immediately and 3 days after intravitreal injection, all study and control eyes were examined by indirect ophthalmoscopy with a 20-D aspherical lens for clear and sharp imaging of the retina and to exclude any possible disease in the vitreous, retina, and choroids.

The appearance of the conjunctiva and cornea in addition to the examination of the anterior chamber and anterior vitreous for evaluation of cellular reaction and flare were inspected by handheld slit lamp under the highest magnification.

Histopathologic Analysis. After the final clinical examination on day 14 (early groups) or by month 3 (late groups), the animals were killed by intraperitoneal injection of a lethal overdose of pentobarbital (100 mg/kg body weight). The eyes were enucleated with careful manipulation to preserve globe integrity. Each eye was immediately placed in neutral formalin solution for light microscopic examination.

The eyes were fixed in neutral formalin solution for 2 days and immersed in 30% sucrose solution for easy dissection for another 48 hours. Then, the enucleated eyes were bisected vertically, dehydrated in a series of 95% ethanol, and embedded in paraffin. The sections were obtained from slices through the whole globe oriented along the optic nerve and medullary ray. Sagittal retinal sections of 5-µm thickness were collected on poly-L-lysine–coated microscopic slides for H&E staining, immunohistochemical analysis with caspase-3 and -9 staining, and TUNEL assay. All slides were examined by light micro-
scope (BX-50; Olympus, Tokyo, Japan) and photographed with a digital video camera (DP-71 CCD; Olympus).

In Situ TUNEL Protocol. DNA nick-end-labeling was performed with an apoptosis detection kit (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit S7101; Chemicon, Temecula, CA). For each rabbit eye, the TUNEL assay was performed on the first section of each hemi-block of tissue. Sections were cut at 5 μm, deparaffinized, and rehydrated through graded concentrations of alcohol. Protein was then digested by treating the tissue slides with proteinase K (00-3011; Zymed, South San Francisco, CA) at room temperature for 15 minutes. The slides were rinsed in distilled water, followed by the quenching of endogenous peroxidase activity with 3% hydrogen peroxide for 5 minutes. The apoptosis detection processing included the application of an equilibration buffer directly to each specimen, followed by 15 to 20 seconds’ incubation. Then, 30 μL of working strength deoxyuridinyl transferase (TdT enzyme) was applied directly to each speci-
men, after which the tissue slides were incubated in a humidified chamber at 37°C for 1 hour. Each specimen was put in a coplin jar containing prewarmed, working-strength stop-wash buffer and incubated for 10 minutes and antitidoxigenin peroxidase was applied to each specimen. The specimens were incubated in a humidified cham-
er for 30 minutes at room temperature. Finally, diaminobenzidine (DAB) was used as the color substrate. Sections were counterstained with 0.5% methyl green and mounted.

Formaldehyde fixed, paraffin-embedded sections from female ro-
dent mammary gland retrieved 3 to 5 days after the weaning of rat pups were obtained from the manufacturer and used as the positive control. The negative control experiments were performed without active TdT but including proteinase K digestion to control for nonspecific incor-
poration of nucleotides or for nonspecific binding of enzyme enzy-
gate.

Quantitative Analysis of TUNEL. For each sample, the num-
ber of TUNEL-positive cells in the outer nuclear layer (ONL) was
determined in three meridian sections through the optic nerve head. Sections were examined at high magnification, and color images were obtained with a digital camera (DP-71 CCD; Olympus). Apopticotic cells were counted in three different microscopic fields of each retinal section (NIS-Elements BR 2.30; Image Analysis System; Nikon, Tokyo, Japan). The number of apoptotic TUNEL-positive cells is expressed as the ratio of TUNEL-labeled nuclei to the total number of nuclei exam-
ined. The mean of the three sections was used as independent data for each eye.

Immunohistochemical Staining. Immunohistochemical staining for caspase-3 and -9 was performed by the standard streptavi-
din-biotin-peroxidase method (cat no. 85-6743, Histostain Plus Rabbit Primary Kit; Zymed). Tissue sections were deparaffinized with xylol and rehydrated with graded alcohol concentrations and then subse-
quently in distilled water. To obtain better and more specific staining, the antigen retrieval method was used as follows: Sections were put in a coplin jar containing citrate buffer (pH 6.0; 15-M103; Bio Optica, Milan, Italy) and irradiated in microwave oven for 4 × 2 minutes at a power of 800 W. Then, the sections were incubated in the same buffer solution for 15 minutes. The specimens were washed in 0.01 M phosphate-buffered saline (pH 7.4)+Tween 20 (PBST; 519G66, Dako, Carpinteria, CA) solution (0.1% solution). After the antigen retrieval, endogenous peroxidase activity was quenched with 3% hydrogen per-
oxide for 10 minutes, and the sections were exposed to blocking serum for 60 minutes to block any nonspecific reaction sites. Caspase-3 (sc-7148, diluted 1:50; rabbit polyclonal antibody; Santa Cruz Biotechno-
ylogy, Santa Cruz, CA) and caspase -9 (rabbit polyclonal antibody, sc-8355, diluted 1:50; Santa Cruz) were then added and the sections were incubated at +4°C overnight. Antibodies were diluted with an antibody diluent solution (cat no. 00-3118). The specimens were rinsed with 0.01 M PBS three times. Tissue sections were then treated with biotin-labeled rabbit anti-goat immunoglobulin G at room temper-
ature for 15 minutes, rinsed with PBST, followed by adding streptavi-
din-biotin-peroxidase complex at room temperature for 15 minutes.

Finally, the sections were stained with DAB chromogen and cover-
slipped. In the negative control experiment, sections were processed using the same protocol as above, except that the primary antibody was replaced by blocking serum. Positive controls were formaldehyde fixed, paraffin-embedded sections from female rodent mammary gland retrieved 3 to 5 days after the weaning of rat pups. Sections were observed with a photomicroscope (BX-50; Olympus) and the immuno-
labeled cells were identified on each section.

Statistical Analysis

Statistical analysis was performed with commercial software (SPSS for Windows, ver. 13.0; SPSS, Chicago, IL). An independent, two-sample test (Mann-Whitney U test) was used to calculate differences in the mean percentage of apoptotic TUNEL-positive cells between the control and any study group or between two study groups. Data in the figures are expressed as the mean ± SD. P < 0.05 was considered statistically significant.

RESULTS

Bevacizumab and pegaptanib sodium were tolerated well. No apparent changes in the retinas were noticed by fundus exami-
nation in all groups at the different times. No conjunctival hyperemia, anterior chamber reaction, or any pupillary abnor-
mality was seen. The vitreous demonstrated no opacities or degeneration.

All study and control eyes were evaluated by H&E staining. At all time points after intravitreal injection of bevacizumab or pegaptanib sodium, the overall appearance of the retina did not differ significantly from the control group. The morpho-
logic appearances of the cornea, iris, retina, choroid, sclera, and optic nerve were normal in both control and study groups.

No sign of retinal necrosis, cystic degeneration, or hypocellu-
arity of the nuclear layer was observed in any of the groups. Some polymorphonuclear leukocytes were noted in all groups, suggesting inflammatory reaction in the limited areas of injec-
tion site at the limbus.

All saline-injected and control eyes showed caspase-3- and -9-positive cytoplasmic staining in the inner and outer limiting

membrane, and inner plexiform layer (Fig. 1B). A similar pat-
tern of staining was observed in eyes in which bevacizumab or pegaptanib sodium had been injected from both the 14-day and 3-month groups (Figs. 1C–H). In the study groups, immunohis-
tochemical staining was also demonstrated in the outer plexi-
form and photoreceptor cell layers.

TUNEL-positive staining was observed in the ganglion cell lay-
er, inner nuclear layer, and retinal pigment epithelium in both early and late-term groups into which bevacizumab or pegaptanib sodium had been injected. A similar pattern of labeling was also observed in eyes in the saline-injection and control groups (Fig. 2). TUNEL staining detected apoptotic cells in the ONL only in the study groups (Figs. 2C–J). In-
creased apoptotic staining in the ONL was more evident in groups 3, 4 (Fig. 2D), and 6 (Figs. 2E, 2F; 1.25-mg day 14 group, 2.5-mg day 14 group, and 0.625-mg three-injection group). Increased apoptosis in photoreceptor cell nuclei was seen at similar levels in all groups that received pegaptanib sodium (Figs. 2G–J). The levels of apoptotic, TUNEL-positive cells are displayed in Figures 3 to 5.

Apoptotic activity, shown by positive TUNEL staining, was analyzed statistically and seen to increase toward higher doses in eyes receiving bevacizumab (Figs. 3, 4). In the bevacizumab-injected groups, 0.625-, 1.25-, and 2.5-mg doses at 14 days and 0.625 mg at 3 months (three injections over 3 months) caused significantly more apoptotic activity when compared to the 0.25-mg dose at day 14 (mean percentage of apoptotic cell counts: 43.0 ± 2.2, 55.3 ± 2.6, 64.5 ± 4.7, 49.8 ± 4.3, and
When compared to the saline-injection group, a single injection of 0.25 mg bevacizumab at day 14 did not cause significant apoptosis \((P = 0.66); 0.625, 1.25, \text{ and } 2.5 \text{ mg bevacizumab at day 14 caused significantly more apoptotic activity when compared with that in the saline-injection group \((P = 0.03, 0.02, \text{ and } 0.02, \text{ respectively). A single dose of } 0.625 \text{ mg bevacizumab evaluated at } 14 \text{ days or at } 3 \text{ months after the injection and at } 3 \text{ months after three injections (one per month) of caused more apoptotic activity than the saline injection \((P = 0.03, 0.02, \text{ and } 0.02, \text{ respectively). The difference in apoptotic activity between the } 0.625- \text{ and } 1.25-\text{mg doses of bevacizumab or between the } 1.25- \text{ and } 2.5-\text{mg doses was statistically significant \((P = 0.02). There was also a statistically significant difference in apoptotic activity between a single injection of 0.625 mg bevacizumab and three injections over three months \((P = 0.02). The lowest dose of 0.15 mg pegaptanib sodium at day 14, which corresponds to the dose used clinically in humans, as well as doses of 0.3 and 0.6 mg pegaptanib sodium at day 14 caused significantly more apoptotic activity than saline \((45.3\% \pm 6.1\%, 49.3\% \pm 4.6\%, 48.5\% \pm 2.6\%, \text{ and } 32.3\% \pm 3.1\%, \text{ respectively; } P = 0.02 \text{ for three doses of pegaptanib compared to saline; Figs. 3, 5). A single injection and three injections over } 3 \text{ months of } 0.15 \text{ mg pegaptanib sodium (}45.8\% \pm 5.7\% \text{ and } 44.3\% \pm 4.0\%; \text{ percentage of mean apoptotic cells, respectively) also caused significant apoptotic activity when compared to that in the saline-injection group \((P = 0.02 \text{ for each comparison). When the pegaptanib sodium groups were com-
Figure 2. Detection of apoptotic cells by TUNEL staining. (A) Sham control. (B) Positive staining was observed in ganglion cell layer (GCL) and inner nuclear layer (INL) in the saline-injection group. (C–F) Apoptosis in the bevacizumab groups. Apoptosis was seen in the GCL, INL and ONL. An evident increase in apoptotic staining in ONL was seen in groups 4 and 6 (2.5 mg/day14 and 0.625 mg/3-injection groups). (G–J) Apoptosis in the pegaptanib sodium group. TUNEL-positive staining was detected in GCL, INL, and ONL. (F, J) TUNEL+/methyl green staining in groups 6 and 11. Bev, bevacizumab; Peg, pegaptanib sodium.
pared to each other, no significant difference in apoptotic activity was found between the different dose groups in contrast to that in the bevacizumab groups. Higher concentrations of pegaptanib sodium did not cause more apoptotic activity than did lower concentrations.

When the pegaptanib sodium groups were compared to the bevacizumab groups, no significant difference in apoptotic activity was observed between the clinical doses (0.625 mg bevacizumab versus 0.15 mg pegaptanib) and between the twofold clinical doses (1.25 mg bevacizumab versus 0.3 mg pegaptanib; \( P < 0.88 \) and \( P < 0.08 \), respectively; Fig. 3). However, apoptotic activity between fourfold clinical doses of both drugs (2.5 mg bevacizumab versus 0.6 mg pegaptanib) was significantly different (\( P < 0.02 \)). In the long-term groups, pegaptanib sodium and bevacizumab did not cause significantly different apoptotic activity in clinical doses when compared to each other (\( P < 0.88 \) and \( P < 0.15 \) for single and three injections, respectively).

**DISCUSSION**

The purpose of our study was to reveal any toxic side effects of bevacizumab and pegaptanib sodium after intravitreal injection, and we evaluated possible apoptosis in photoreceptor cells for different doses of tested agents. Overall, no toxicity-related adverse effects were observed by clinical and routine light microscopic assessment (data not shown), and no inflammatory reaction was demonstrated in the experimental groups. In our previous study, we reported that intravitreal bevacizumab injection was related to mitochondrial disruption in the inner segments of photoreceptors documented by electron microscopy and also increased apoptosis in inner layers of the retina by caspase-3, caspase-9, and bax/bcl-2 immunostaining, but we did not perform quantitative evaluation of apoptosis in the previous study. In the present study, we used the TUNEL method for detection of DNA fragmentation in nuclei of apoptotic cells in nuclear layers. Caspase-3 and -9 staining is a marker of apoptosis that is based on cytoplasmic localization in cells containing activated caspase and shows good correlation with morphologic apoptosis demonstrated by the TUNEL method. Statistical analysis was performed for the sections stained with the TUNEL method.

In our study, we also detected apoptosis in the control eyes. It seems that the apoptotic process continues physiologically during life. Although there is no study in which the apoptotic process was specifically evaluated by the TUNEL method in normal rabbit retina, it has been demonstrated in retinal cells in the control eyes of several experimental animal studies. Apoptotic activity was shown in ganglion cells and inner and

**FIGURE 3.** The distribution of apoptotic cells in the ONL for all groups (median cell count in box-and-whisker format). Bevacizumab-treated groups: 1: 0.25 mg/day14; 2: 0.625 mg/day 14; 3: 1.25 mg/day 14; 4: 2.5 mg/day 14; 5: 0.625/one injection, 90 days; and 6: 0.625 mg/three injections, 90 days. Pegaptanib sodium treated groups: 7: 0.15 mg/day 14; 8: 0.3 mg/day 14; 9: 0.6 mg/day 14; 10: 0.15/one injection, 90 days; and 11: 0.15 mg/3 injections, 90 days; group 12: saline control; and group 13: sham control.

**FIGURE 4.** Comparison of the percentage of apoptotic cells (mean ± SD) in the ONL of the control and saline-injection groups with the bevacizumab-injected groups. G1: 0.25 mg/day 14; G2: 0.625 mg/day 14; G3: 1.25 mg/day 14; G4: 2.5 mg /day 14; G5: 0.625/one injection, 90 days; and G6: 0.625 mg/three injections, 90 days. *Statistically significant when compared to the saline-injection group (\( P < 0.05 \)).

**FIGURE 5.** Comparison of the percentage of apoptotic cells (mean ± SD) in the ONL of the control and saline groups with the pegaptanib sodium-injected groups. G7: 0.15 mg /day 14; G8: 0.3 mg/day 14; G9: 0.6 mg/day 14; G10: 0.15/one injection, 90 day; and G11: 0.15 mg/three-injection, 90 days. *Statistically significant when compared to the saline-injection group (\( P < 0.05 \)).
outer nuclear cell layers in the control groups of the porcine retina-pigment epithelium-choroid culture.\textsuperscript{11} the apoptotic process was also shown in normal primate photoreceptor cells with lower levels.\textsuperscript{24} In our previous study we detected apoptosis in control rabbit eyes with caspase and bax/bcl-2 immunostaining.\textsuperscript{15} Whether nonspecific background staining increased the apoptotic cell count in the sham and saline control eyes remains to be answered in future studies.

Our finding that bevacizumab caused apoptosis at higher doses may suggest that increasing the dosage with intravitreal bevacizumab can cause greater toxic effects on retinal neuronal cells. Similar dose-related adverse effects were not found for pegaptanib sodium. Pegaptanib sodium–related apoptosis was similar among the clinical, twofold, and fourfold dose groups. This dose-independent effect remains to be investigated. When the apoptotic activity of bevacizumab and pegaptanib was compared, it was seen that there was no difference in the toxicity of these agents at clinical and twofold doses, but bevacizumab caused more evident toxicity in comparison to pegaptanib when fourfold doses of both agents were used. After pegaptanib injection, other VEGF isoforms may protect the retina when only the 165 isoform was blocked. Another explanation may be a faster clearance of pegaptanib sodium from the vitreous. However, pegaptanib sodium in clinical doses was also seen to evoke apoptosis when compared with the saline control. The other finding from the present study was that the commonly used clinical dose of bevacizumab corresponding to 0.625 mg in rabbit eyes caused a significant increase in apoptosis in comparison to the saline injection. In addition, repeated injections of the same dose of bevacizumab were related to more apoptosis than was a single dose. Thus, in clinical practice it should be borne in mind that bevacizumab should be used in the lowest possible doses.

Anti-VEGF activities of bevacizumab and pegaptanib together with ranibizumab have been compared in vitro. Pegaptanib showed no effect, whereas the others neutralized VEGF completely at clinical doses.\textsuperscript{10} Pegaptanib sodium has been verified to be effective in patients with ARMD with multicenter clinical trials, but the results in these trials remained in the shadow of results of trials of complete VEGF inhibitors. When considering the VEGF-related physiological functions and possible toxic effects of pan-VEGF antagonism, pegaptanib sodium was regarded as more reliable for both ocular and systemic safety. It is not presently clear whether related results from in vitro or experimental animal studies, including the present study might help to explain clinical experience with pegaptanib sodium.

Several reports have demonstrated that VEGF not only induces angiogenesis, but also works as a survival factor for tumor and endothelial cells, protecting from apoptosis.\textsuperscript{33} Withdrawal of VEGF leads to endothelial cell apoptosis in vitro and in vivo.\textsuperscript{25} Additional VEGF functions under investigation include retinal leukostasis and neuroprotection.\textsuperscript{27} It has been reported that VEGF, besides its neuroprotective effects, has a neurotrophic influence, by stimulating axonal outgrowth from dorsal root ganglia or superior cervical ganglia in culture.\textsuperscript{29,30} In the adult retina, VEGF is expressed by retinal pigment epithelial cells as well as by neuronal and glial cells in the ganglion cell layer and inner nuclear cell layer.\textsuperscript{31} VEGF receptor (VEGFR)-2 is expressed by adult photoreceptor cells and Müller cells. It has been suggested that VEGF and their receptors provide an endogenous mechanism that may protect the retina against damage of different origins and may also promote axonal regeneration in central nervous system (CNS) lesions.\textsuperscript{29} It has also been suggested that tissue inhibitor of matrix metalloproteinase-1 and VEGF in combination promote the survival of photoreceptor cells in rat retinal explants, possibly by affecting a caspase-3 signaling pathway.\textsuperscript{32} Retinal neuronal cells as well as Müller cells require VEGF as a survival factor under normal conditions. Dependence on VEGF signaling may be due to the high metabolic rates of retinal photoreceptor cells and their extreme sensitivity to hypoxia.\textsuperscript{35} Therefore, in situations especially where the retina is under threat of hypoxia, a complete deficiency of the physiological level of endogenous VEGF may be critical for photoreceptor cells.

In a mouse model, systemic neutralization of VEGF led to significant cell death in the inner and outer nuclear cell layer at 14 days as well as loss of visual function.\textsuperscript{54} Cell death was more evident in the ONL where a high number of photoreceptor cells are present. It has been suggested that the physical association between the photoreceptors that express VEGF receptors and the Müller cells that express VEGF points to a paracrine interaction in which Müller cells provide VEGF as a neurotrophic signal to photoreceptors. VEGF blockade has also been demonstrated to cause ganglion cell apoptosis in a rat model.\textsuperscript{55}

The neuroprotective effect of VEGF has been suggested to involve suppression of cell-death pathways mediated by caspase-3, which plays a key role in apoptosis and is induced by caspase 9. Evidence suggests that VEGF exerts its antiapoptotic action through VEGFR-2 receptors and the phosphatidylinositol 3'-kinase/Akt signal transduction system. The serine-threonine protein kinase Akt contributes to the inhibition of apoptosis by activating antiapoptotic proteins through the transcription factor NFκB and inhibiting proapoptotic signaling by Bad, caspase-9, and other effectors. Akt inhibits the proapoptotic Bcl-2 family member Bad and caspase-9. The proapoptotic protein Bad acts at the surface of the mitochondrial membrane to decrease the mitochondrial transmembrane potential and promotes leakage of cytochrome c. Released cytochrome c complexes with and activates Apaf-1, which binds to procaspase-9, and converts this enzyme into its proteolytically active form. Caspase-9 leads to apoptosis through nuclear damage (DNA fragmentation, DNA mutations). The inhibition of Bad and caspase-9 by Akt results in proteolytic cascade blockage and prevents caspase-3-mediated DNA fragmentation. However, further studies are needed to delineate the additional mechanisms by which VEGF exerts its antiapoptotic effects on retinal neuronal cells.\textsuperscript{36}

Our results regarding the effect of anti-VEGF therapy on apoptosis are supported by those in previous studies. Increased apoptotic cell death detected at inner and outer photoreceptor layers in our study is consistent with the findings that bevacizumab immunoreactivity can be found in several retinal cell types 14 days after injection, the most prominent of them being rod photoreceptor outer segments and endothelial cells of blood vessels.\textsuperscript{3}

An electron microscopy study showed that intravitreal bevacizumab caused ultrastructural changes in the choriocapillaris with significant reduction of choriocapillaris endothelial cell fenestration in primate eyes. Atrophy of choriocapillaris or loss of its endothelial cell fenestrations impairs nutritional support, which may lead to functional and morphologic damage to the RPE and photoreceptors, with particular adverse effects if the macular region is affected.\textsuperscript{14} In addition, in a recent study in which a retinal neovascularization model was developed, it has been suggested that caution may be warranted in the treatment of patients with acute or severe retinal neovascularization by anti-VEGF drugs such as bevacizumab to prevent capillary nonperfusion and macular ischemia.\textsuperscript{3} The established neuroprotective and neurotrophic effect of VEGF may explain an increase in apoptosis in retinal neuronal cells by intravitreal anti-VEGF injections.

Bevacizumab, ranibizumab, and pegaptanib significantly suppressed choroidal endothelial cell proliferation in vitro when used at the currently recommended intravitreal dose.
However, when used at the established dose, none of the drugs was superior over the others with respect to endothelial cell growth inhibition. No cytotoxicity was detectable for pegaptanib and bevacizumab at the respective concentrations that included the clinical dose for both drugs. In another study, it was suggested that bevacizumab, administered at or above the dose normally used in clinical practice, is safe in the short term for retina pigment epithelium (RPE) cells, rat retinal neurosensory cells, and human microvascular cells in vitro. However, our results suggest that intravitreal administration of high doses of anti-VEGF agents does matter, especially with bevacizumab, when safety is evaluated in regard to apoptosis. When apoptosis in the retina was evaluated in porcine retinal RPE-choroid cultures exposed to bevacizumab (concentrations up to five times higher than those used clinically) for 3 days, no increase in apoptotic cells in the ganglion cell layer or RPE-choroid was observed by TUNEL labeling. In contrast to these findings, our results showed increased apoptosis at higher doses (especially at a fourfold higher dose than that used clinically) in an experimental clinical rabbit study.

On the other hand, no toxic effects of intravitreal bevacizumab on mouse retina, as examined by histologic and TUNEL apoptosis analysis, were found even at high doses in a study published very recently. Although the results from this study are contrary to our results, differences in species may compromise interpretation for comparison of conflicting results. This monoclonal antibody has been shown to inhibit tumor growth in a dose-dependent manner in various animal models. Bevacizumab has been reported to bind to primate VEGF and, with lower affinity, to rabbit VEGF, but does not bind to murine VEGF, because of an amino acid substitution in the bevacizumab-binding site. There is a controversy about the efficacy of anti-VEGF antibodies in murine models. However, some studies suggest that bevacizumab may bind to murine VEGF, at least with lower affinity. Differences in tissue response to intravitreal bevacizumab in different species may result from binding differences of humanized anti-VEGF to species related VEGF, differences in elimination half-life, and differences in penetration of bevacizumab to retinal layers.

In our study, we assessed the effects of a single injection or three injections (one per month for 3 months) of bevacizumab or pegaptanib sodium on apoptosis. A significant increase in programmed cell death in sensory-neural retina with anti-VEGF therapy in experimental rabbit eyes may be of some importance for patients with AMD, diabetic retinopathy, or macular edema caused by several diseases in which anti-VEGF therapy may last many months. The results should be extrapolated carefully to clinical practice. As a result, intravitreal injection of bevacizumab and pegaptanib sodium as a single high dose or repeated injections of clinical doses may increase apoptosis in retinal photoreceptor cells. Further studies are needed to test our results and clarify the long-term toxic effect of anti-VEGF treatments on retinal neuronal cells especially at ultrastructural levels.

References


