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RETINAL DISORDERS

Are intravitreal bevacizumab and ranibizumab effective in a rat model of choroidal neovascularization?

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Abstract
Background Vascular endothelial growth factor (VEGF) is an important stimulator of choroidal neovascularization (CNV). Bevacizumab (Avastin), ranibizumab (Lucentis) and pegaptanib sodium (Macugen) are anti-VEGF medications that have been used in the treatment of CNV. The purpose of our study is to evaluate the efficacy and safety of intravitreal injections of bevacizumab, ranibizumab and pegaptanib sodium in the treatment of CNV in a rat model.
Methods Multiple CNV lesions were induced by laser photocoagulation of the retina in Brown-Norway rats. After 3 weeks, 17 rats were divided into three groups and received intravitreal injections of bevacizumab, ranibizumab or pegaptanib sodium in different dosages. The lesions were evaluated by fluorescein angiography 1, 7, 14, and 28 days later to assess the efficacy of these medications.
Results Different doses of bevacizumab did not show any effect on stopping the leakage on fluorescein angiography on days 1, 7, 14, and 28. Ranibizumab and pegaptanib sodium did not stop the leakage of CNV either. No angiographic or histopathologic toxicity was observed.
Conclusions These three anti-VEGF agents did not show any therapeutic effect on stopping CNV leakage in rats. Previous experiments with ranibizumab in monkeys resulted in a significant decrease in leakage of CNV. The difference may be due to the fact that both ranibizumab and bevacizumab are humanized and species-specific. There are several studies evaluating the effect of bevacizumab in non-primates. Since bevacizumab is humanized, the results of studies on non-primates may not be similar to humans and non-human primates.

Keywords Anti-VEGF · Choroidal neovascularization · Angiogenesis · Age-related macular degeneration

Introduction

Vascular endothelial growth factor (VEGF) plays a major role in the pathophysiologic basis of the angiogenesis. In the eye, VEGF may lead to the development and progression of neovascular age-related macular degeneration (AMD), proliferative diabetic retinopathy, retinopathy of prematurity, and other neovascular disorders of the eye. Levels of VEGF in the eye have been shown to rise in diseases that involve intraocular neovascularization [1]. Inhibiting VEGF-dependent angiogenesis and decreasing vascular permeability are potential treatments for neovascular AMD. As a humanized monoclonal antibody to VEGF, bevacizumab (Avastin™; Genentech, Inc., South San Francisco, CA, USA), a FDA-approved medication for metastatic colon cancer and breast cancer, can reduce the leakage of choroidal neovascularization (CNV) in AMD [2]. Ranibizumab (Lucentis™, Genentech, Inc.), similar to bevacizumab, a chemically modified, 48 KD humanized fragment antigen binding (Fab) of a monoclonal anti-VEGF antibody, and pegaptanib sodium (Macugen™, Eyetech Pharmaceuticals/OSI Pharmaceuticals, Long Island, NY, USA) a 28-nucleotide RNA aptamer specific for the VEGF165 isoform, are both approved for treatment of AMD by the FDA. They are significantly more expensive than bevacizumab. However, so

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far the safety and efficacy of bevacizumab has not been directly compared to ranibizumab.

There are approximately 10 million patients affected by age-related macular degeneration in the United States, and approximately 10% of them have the wet (neovascular) form of AMD [3]. Therefore, there are a large number of AMD patients that potentially may require treatment. It is important to evaluate the efficacy and safety of bevacizumab for ophthalmic administration. A head-to-head clinical trial—Comparison of Age-related Macular Degeneration Treatments Trials (CATT)—is designed to compare the safety and efficacy of bevacizumab to ranibizumab. However, clinical trials are lengthy, and the result of the trials may not be available for a few years. Although no retinal toxicity has been reported after intravitreal injection of bevacizumab, the safety needs further studies [4].

There are several studies regarding ocular effects of bevacizumab on experimental animals, such as rabbits [5], rats [6, 7], mice [8] and monkeys [9]. We have studied ranibizumab in a monkey model of choroidal neovascularization [10], and the effect was similar to the human response to ranibizumab. There is a controversy about the efficacy of anti-VEGF antibodies in murine models [11]. Our study is designed to answer whether ranibizumab or bevacizumab are effective in a laser-induced rat model of choroidal neovascularization.

A rat model of laser-induced CNV has been utilized for the evaluation of experimental treatments of AMD, such as verteporfin in photodynamic therapy (PDT) [12], intravitreal triamcinolone acetonide [13], angiostatin [14], etc. The result of PDT in a rat model was comparable to human response [12]. The rat model of laser-induced CNV has a high reproducibility and reliability. The neovasculatures in rats leak for 8 weeks or longer [15]. Moreover, this model is inexpensive and easy to use. In order to compare the ability of these anti-VEGF agents in stopping leakage of choroidal neovascularization, we investigated laser induced rat model for evaluation of three anti-VEGF medications: bevacizumab, ranibizumab and pegaptanib sodium.

Material and methods

Induction of choroidal neovascular membranes

All experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Yale University Institutional Animal Care and Use Committee on Animal Research. Brown-Norway rats (250 g–300 g) were purchased from Harlan Sprague Dawley, Inc, and anesthetized with a 0.2 ml-0.3 ml of a 1:1 mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) through intraperitoneal injection. Pupils were dilated with a topical application of 2.5% phenylephrine and 1.0% tropicamide. An argon green laser (Model Ultima 2000 SE, Coherent Medical Laser) was used to induce six or seven laser spots in each eye around the optic nerve. The wavelength was 532 nm, and the spot size was 100 µm. Power delivered ranged from 160 mW to 170 mW, applied for 0.1 sec. A bubble or a small subretinal hemorrhage (diameter <1 mm) at the laser spot indicated rupture of the Bruch's membrane [12]. The formation of CNV was confirmed by fluorescein angiography and fundus photography. Fluorescein angiography 3 weeks after laser showed early hyperfluorescence with late leakage at the site of the laser injury (Fig. 1a).

Fluorescein angiography

Rats were anesthetized, and their pupils were dilated with 2.5% phenylephrine and 1.0% tropicamide. Fluorescein angiography was performed using a digital fundus camera (model TRC 50 EX; Topcon, Paramus, NJ, USA) and a 50° angle of view. 1 ml of 10% sodium fluorescein was injected intraperitoneally [12]. Fluorescein angiography was performed 3 weeks after laser photocoagulation to confirm the growth of CNV, and also on days 1, 7,14, and 28 after intravitreal injection of bevacizumab, ranibizumab or pegaptanib sodium (Table 1).

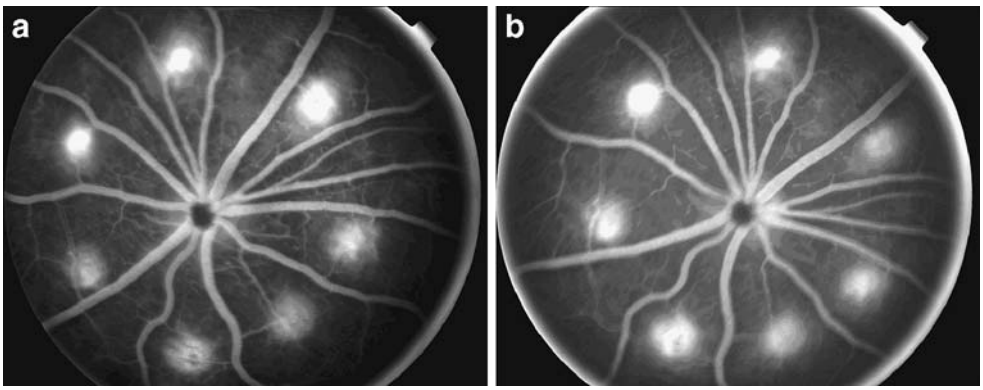

Fig. 1 Fluorescein angiography of a control eye. **a** Late phase of fluorescein angiogram of a Brown-Norway rat 3 weeks after laser photocoagulation (day 0), shows the hyperfluorescence at the site of laser injury. **b** The late phase of fluorescein angiogram of the same eye 7 weeks after laser photocoagulation (day 28), shows the consistent leakage of CNV

Table 1 Experiment design (CNV: choroidal neovascularization; VEGF: vascular endothelial growth factor)

Date of test	Action
Day -21	Argon green laser
Day -1	Fluorescein angiography to confirm the presence of CNV
Day 0	Injection of anti-VEGF agent
Day 1	Fluorescein angiography
Day 7	Fluorescein angiography
Day 14	Fluorescein angiography
Day 28	Fluorescein angiography

Dosage and administration of medications

Eyes were randomly assigned to three groups; group 1 received intravitreal injection of bevacizumab, group 2 received ranibizumab, and group 3 received pegaptanib sodium. The average volume of human vitreous is about 4.0 ml, while the volume of rat vitreous varies from 56 µl [16] to 12 µl [17]. The dose equivalent to human dose was calculated as about 1/30–1/70 of humans. The doses of bevacizumab and ranibizumab that were used in this experiment included not only the equivalent dose (similar vitreous concentration) to humans, but also other doses. The dosages of bevacizumab were 0.025 mg (1 µl), 0.05 mg (2 µl), 0.125 mg (5 µl) and 0.25 mg (10 µ), ranibizumab 0.02 mg (2 µl), 0.05 mg (5 µl) and 0.1 mg (10 µl), and pegaptanib sodium 0.01 mg (3 µl). Intravitreal injections were performed by inserting a 31-gauge needle, attached to a 10-µl Hamilton syringe. The lesions were evaluated by fluorescein angiography 1, 7, 14 and 28 days following intravitreal injection to assess the efficacy of these medications. Six eyes were left untreated as an internal control after the laser injury. Fluorescein angiography was performed on these control eyes once per week for 7 weeks after laser photocoagulation. Three eyes from bevacizumab and three eyes from ranibizumab groups were enucleated 14 days after intravitreal injection, and stained by hematoxylin and eosin for histopathologic examination.

Results

Fluorescein angiography

The fluorescein angiography in control eyes showed early hyperfluorescence with late-phase leakage at the site of laser injury 7 days after laser photocoagulation, which reached its peak on about day 21 (Fig. 1a). The hyperfluorescence of CNV was still present 50 days after laser (end point of our experiment).

Following administration of various dosages of bevacizumab, none of the doses showed any effect in stopping the angiographic leakage on fluorescein angiogram on days 1, 7, 14, and 28. Figures 2 and 3 show the fluorescein angiogram of bevacizumab treated eyes in dosage of 0.05 mg and

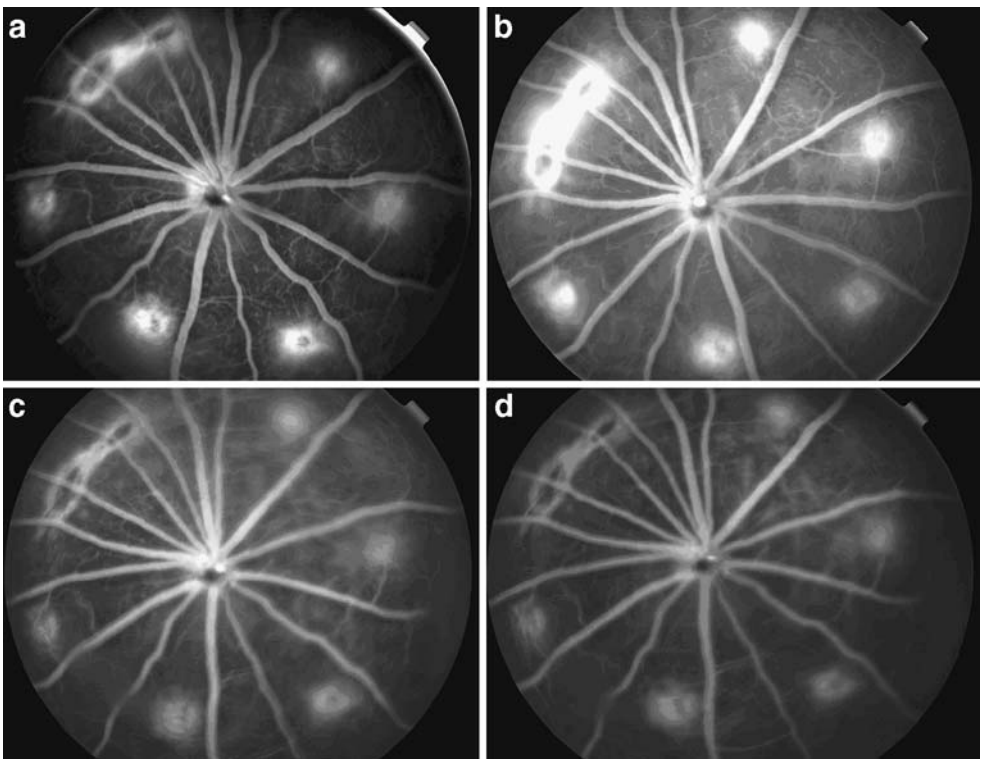

Fig. 2 Intravitreal injection of bevacizumab (0.05 mg/2 µl). **a** Fluorescein angiogram of a Brown-Norway rat at the baseline (before injection of bevacizumab). The angiogram shows leakage consistent with choroidal neovascular membrane. **b**,**c**,**d** The late phase of fluorescein angiogram of the same eye 7, 14, and 28 days after intravitreal bevacizumab injection respectively. No significant therapeutic effect was noted

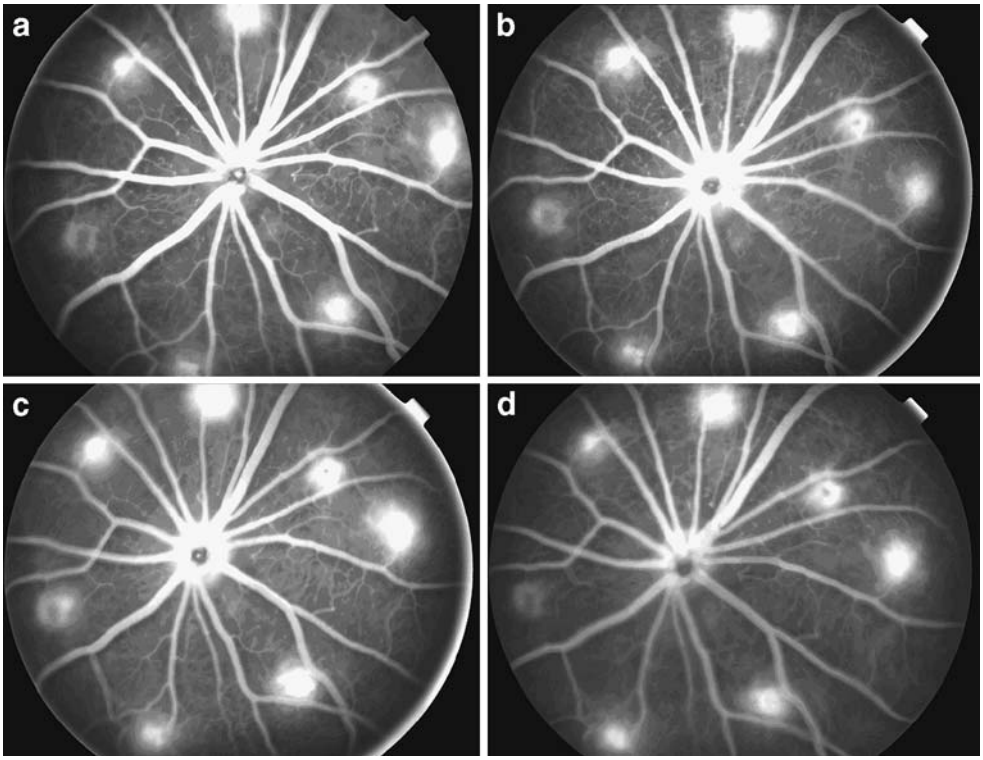

Fig. 3 Intravitreal injection of bevacizumab (0.25 mg/10 μl). **a** Fluorescein angiogram of a Brown-Norway rat at the baseline (before injection of bevacizumab). **b**,**c**,**d** The late phase of fluorescein angiogram 7, 14, and 28 days after bevacizumab treatment respectively. No significant therapeutic effect was noted

0.25 mg respectively. Ranibizumab (Fig. 4) and pegaptanib sodium (Fig. 5) did not stop the leakage of CNV either. No fundoscopic, angiographic, or histopathologic side effect was observed in rats.

Histopathologic examination

In control eyes, which did not receive any anti-VEGF medication, a choroidal neovascular membrane with new

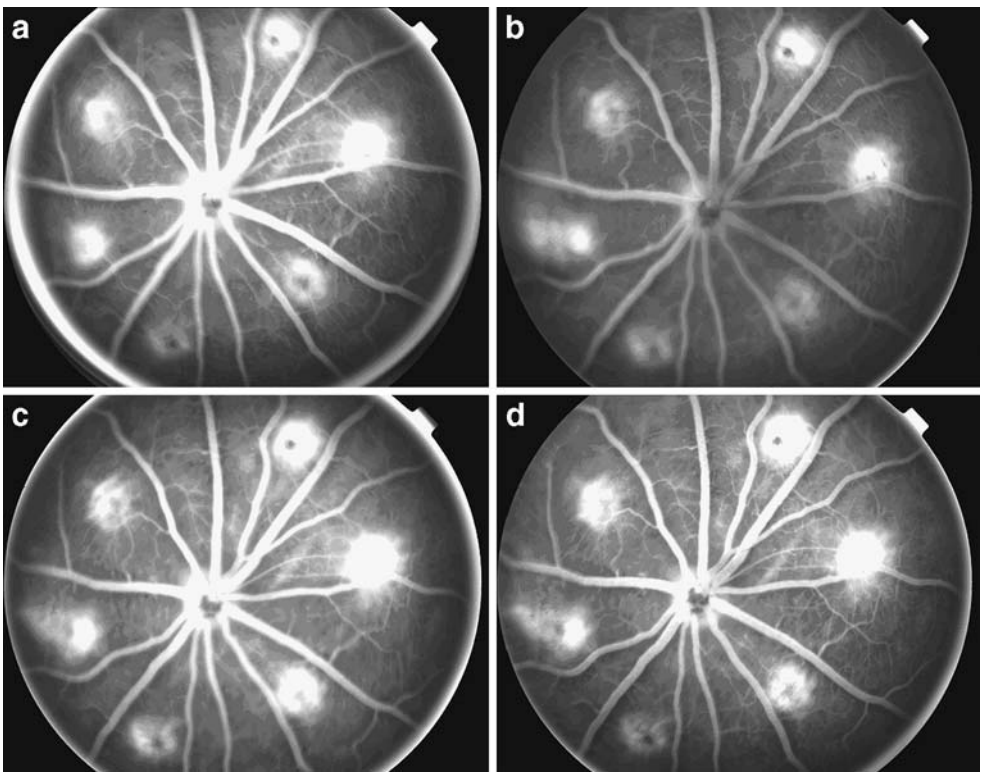

Fig. 4 Intravitreal injection of ranibizumab (0.02 mg/2 μl). **a** Fluorescein angiogram of a Brown-Norway rat at the baseline (before injection of ranibizumab). **b**,**c**,**d** The late phase of fluorescein angiogram 7, 14, and 28 days after bevacizumab treatment respectively. No significant therapeutic effect was noted

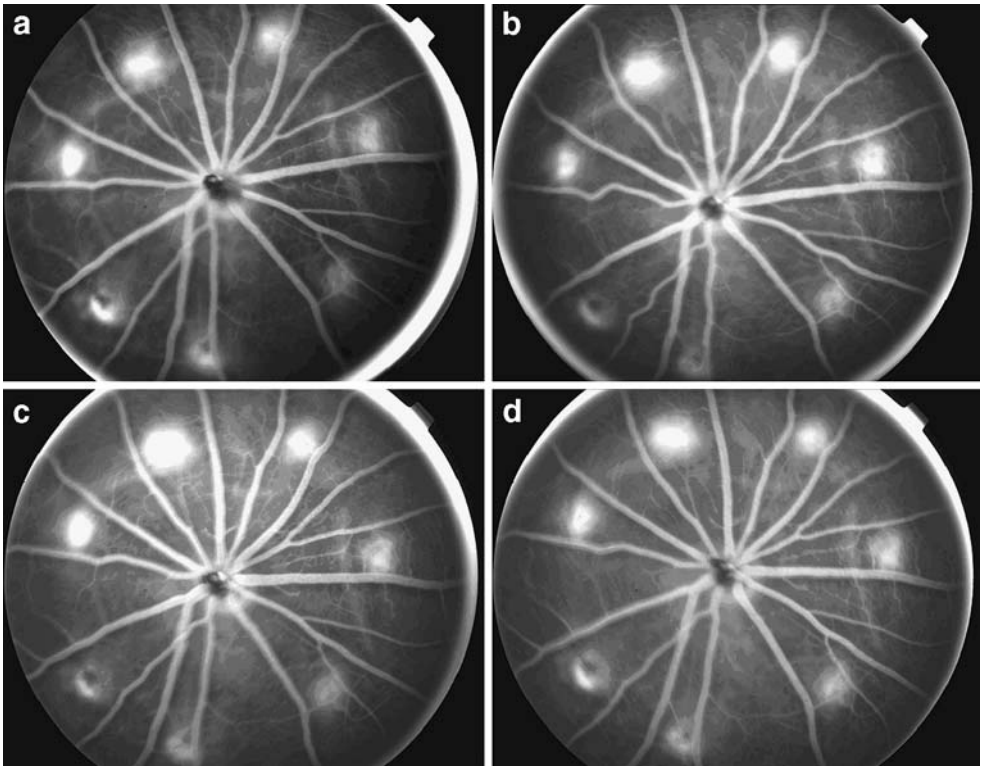

Fig. 5 Intravitreal injection of pegaptanib sodium (0.01 mg/3 μl). **a** Fluorescein angiogram of a Brown-Norway rat at the baseline (before injection of pegaptanib sodium). **b**,**c**,**d** The late phase of fluorescein angiogram 7, 14, and 28 days after bevacizumab treatment respectively. No significant therapeutic effect was observed in the rat model of choroidal neovascularization

vessels and hemosiderin-laden macrophages was seen on day 14 (Fig. 6a). This choroidal neovascular membrane progressed over time to become more fibrotic on day 28. At this time, the membrane was hypocellular, with limited inflammatory response. In the treatment group, 14 days following intravitreal injection of bevacizumab or ranibizumab, the new vessels with patent lumen still existed in the site of laser injury (Fig. 6b,c), No inflammatory reaction related to the injection of medications was found. No other retinal and choroidal changes were noted.

Discussion

Angiogenesis, the process by which new capillaries originate from existing vessels, is a requirement for the establishment of the vascular supply in both normal and abnormal tissues. VEGF has the ability to promote growth of vascular endothelial cells derived from vessels [18].

VEGF is an endothelial cell-specific molecule that acts through two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) [19, 20]. With activation of VEGF,

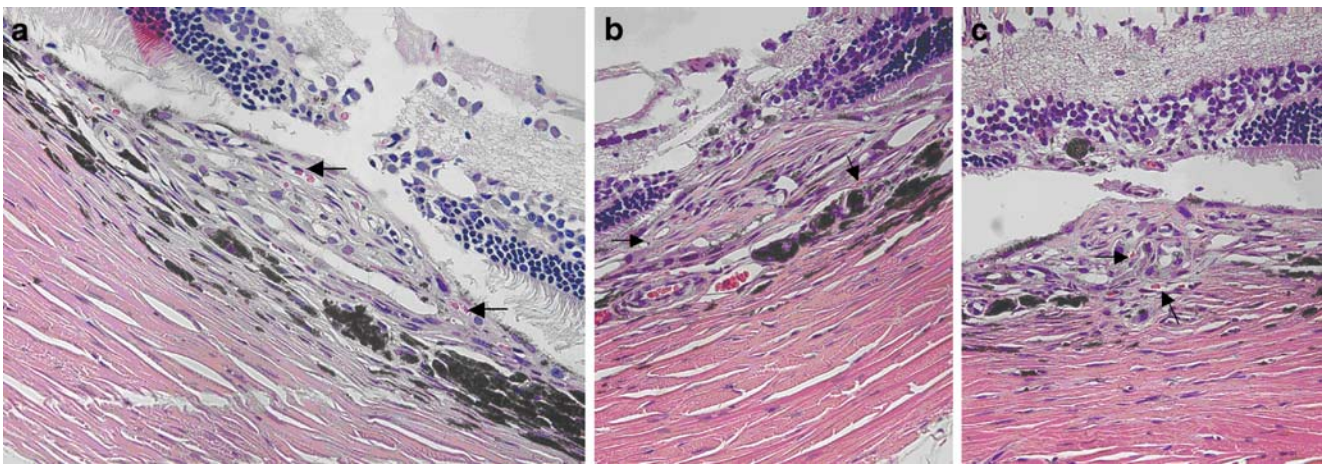

Fig. 6 Hematoxylin and eosin staining of CNV on day 14; control eye (**a**), bevacizumab (**b**),and ranibizumab (**c**). **a** Control; pathologic blood vessels are present (*arrows*) in the area of CNV in the control eye. **b** Bevacizumab; neovascularization is present (*arrows*) in the area of CNV 14 days post injection of bevacizumab (0.25 mg/10 μl). **c** Ranibizumab; pathological blood vessels are also present (*arrows*) in the area of CNV 14 days post injection of ranibizumab (0.1 mg/10 μl). The size and cellular structure of the CNV in the control eye, bevacizumab- and ranibizumab-treated eyes were similar

endothelial cells proliferate, and migrate from existing normal choroidal vessels and develop complicated vascular networks. Without presence of pericytes, these new vessels lack the barrier function of mature endothelial cells. Anti-VEGF agents inhibit this VEGF-dependent proliferation of vessels. Although the pathogenesis of CNV is not fully understood, the blockage of VEGF receptor signaling is responsible for the inhibition of CNV. Thus VEGF is an important stimulator of CNV [21].

Following years of clinical trials, anti-VEGF agents have been proven to be beneficial for the treatment of AMD. Bevacizumab has been approved by the FDA as a treatment for metastatic colorectal and lung cancer in combination with chemotherapy. Also, it has recently been approved for the treatment of breast cancer. Currently, bevacizumab is injected intravitreally as an off-label treatment of neovascular AMD. Similar to bevacizumab, ranibizumab is a humanized anti-VEGF antibody. In a previous study, intravitreal injections of ranibizumab administered every 2 weeks in a laser-induced CNV model in cynomolgus monkeys showed no significant toxic effect, and prevented formation of clinically significant CNV [10]. These results were very similar to the human response in clinical settings. Manzano et al. reported that topically administered bevacizumab limits corneal neovascularization following chemical injury in a rat model [6]. If bevacizumab could interact with rat VEGF, rat would be a good CNV model to evaluate the ocular response to bevacizumab.

There are a few reports regarding the effect of bevacizumab on corneal neovascularization in murine models. Bock et al. reported that systemic or topical application of bevacizumab can significantly inhibit inflammation-induced angiogenesis in the cornea of mice, and bevacizumab binds mouse VEGF-A by Western blot, ELISA, and plasmon resonance assay [8]. Manzano et al. evaluated the effect of topical bevacizumab on a rat model of corneal neovascularization. They applied silver nitrate sticks to cauterize the cornea in Long Evans rats in order to induce neovascularization of the cornea. Topical bevacizumab 4 mg/ml limited corneal neovascularization in rats [6]. Utilizing a similar model of corneal neovascularization in Wistar rats, Barros et al. found subconjunctival injection of bevacizumab 0.02 ml can significantly inhibit corneal angiogenesis [7].

Bevacizumab is a humanized variant of the anti-human VEGF-A monoclonal antibody A.4.6.1, which was derived using hybridoma technology by immunizing mice with human VEGF as originally described by Kim et al. [22]. Since bevacizumab is humanized, it is species-specific. Fuh et al. reported that the structure of the protein does not allow bevacizumab to bind with murine VEGF-A [23]. In a recently published paper, Yu et al. [11] detected no measurable interaction between mouse VEGF and bevacizumab by BIAcore. Bevacizumab also failed to inhibit mouse VEGF-stimulated endothelial cell proliferation. Even at a very high dose, there was not a significant inhibition of mouse VEGF-A by bevacizumab. Additionally, an in vivo study showed that bevacizumab did not inhibit laser-induced choroidal neovascularization in a mouse model, and had no effect on melanoma growth in the same animal [11].

Intravitreal bevacizumab, ranibizumab and pegaptanib sodium were injected to study the safety and efficacy of these medications on a rat model of choroidal neovascularization. In contrast to the corneal studies by Bock et al. and Manzano et al., our results showed that these medications did not stop the leakage of laser-induced choroidal neovascularization in rats.

Manzano et al. [6] used topical bevacizumab 4 mg/ml drops twice a day for 7 days. In our study we used single injection, and the maximum concentration of bevacizumab in the vitreous of rats was about 4 mg/ml. Additionally, the technique and mechanism of induction of neovascularization is site-specific and model-dependent [24]. VEGF receptor expressed in different cells may act differently. The immune response to intravitreal injection of a drug may be different from the immune response to topical administration of the same medication. Since the blood ocular barrier may modify the immune response to intravitreal injections, activation of the rat immune system to the topical administration of humanized antibody may explain the difference in response to anti-VEGF therapy between corneal neovascularization and choroidal neovascularization.

Our study has a few limitations, including the small number of experimental animals and a short follow-up. We did not study the effect of multiple injections on the choroidal neovascular membrane in this group of rats.

In conclusion, our in vivo study suggests that bevacizumab and ranibizumab do not inhibit leakage of laser-induced choroidal neovascularization in rats. Our results suggest that the response to anti-VEGF therapy may be species- and site-specific. Therefore, the effect of VEGF inhibition varies in different neovascular animal models. There are several studies regarding safety, efficacy and pharmacokinetic of bevacizumab in non-primates. The results of experiments in non-primates may not be similar to primates, and those results should be interpreted with caution.

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References

1. Kliffen M, Sharma HS, Mooy HS, Kerkvliet CM, de Jong PTVM (1997) Increased expression of angiogenic growth factors in age-related maculopathy. Br J Ophthalmol 81:154–162
2. Bashshur ZF, Haddad ZA, Schakal A, Jaafar RF, Saab M, Noureddin, BN (2008) Intravitreal bevacizumab for treatment of

neovascular age-related macular degeneration: a one-year prospective study. Am J Ophthalmol 145(2):249–256
3. Klein R, Klein BE, Jensen SC, Meuer SM (1997) The five-year incidence and progression of age-related maculopathy: the Beaver Dam Eye Study. Ophthalmology 104:7–21
4. Weiss M, Roth DB, Prenner JL (2007) The incidence of systemic adverse events in patients treated with intravitreal bevacizumab (Avastin). Invest Ophthalmol Vis Sci 48:4939
5. Ameri H, Chader GJ, Kim J, Sadda SR, Rao NA, Humayun MS (2007) The effects of intravitreous bevacizumab on retinal neovascular membrane and normal capillaries in rabbits. Invest Ophthalmol Vis Sci 48:5708–5715
6. Manzano RP, Peyman GA, Khan P, Carvounis PE, Kivilcim M, Ren M, Lake JC, Chévez-Barrios P (2007) Inhibition of experimental corneal neovascularization by bevacizumab (Avastin). Br J Ophthalmol 91:804–807
7. Barros LM, Belford R Jr (2007) The effect of the subconjunctival injection of bevacizumab (Avastin) on angiogenesis in the rat cornea. An Acad Bras Cienc 79:389–394
8. Bock F, Onderka J, Dietrich T, Bachmann B, Kruse FE, Paschke M, Zahn G, Cursiefen C (2007) Bevacizumab as a potent inhibitor of inflammatory corneal angiogenesis and lymphangiogenesis. Invest Ophthalmol Vis Sci 48:2545–2552
9. Heiduschka P, Fietz H, Hofmeister S, Schultheiss S, Mack AF, Peters S, Ziemssen F, Niggemann B, Julien S, Bartz-Schmidt KU, Schraermeyer U, The Tübingen Bevacizumab Study Group (2007) Penetration of bevacizumab through the retina after intravitreal injection in the monkey. Invest Ophthalmol Vis Sci 48:2814–2823
10. Krzystolik MG, Afshari MA, Adamis AP, Gaudreault J, Gragoudas ES, Michaud NA, Li W, Connolly E, O'Neill CA, Miller JW (2002) Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 120:338–346
11. Yu L, Wu X, Cheng Z, Lee CV, LeCouter J, Campa C, Fuh G, Lowman H, Ferrara N (2008) Interaction between bevacizumab and murine VEGF-A: A reassessment. Invest Ophthalmol Vis Sci 49:522–527
12. Zacks DN, Ezra E, Terada Y, Michaud N, Connolly E, Gragoudas ES, Miller JW (2002) Verteporfin photodynamic therapy in the rat model of choroidal neovascularization: angiographic and histologic characterization. Invest Ophthalmol Vis Sci 43:2384–2391
13. Ciulla TA, Criswell MH, Danis RP, Hill TE (2001) Intravitreal triamcinolone acetonide inhibits choroidal neovascularization in a laser-treated rat model. Arch Ophthalmol 119:399–404
14. Terada Y, Michaud NA, Connolly EJ, Lane A, Ohtsuki H, Gragoudas ES, Miller JW (2003) Enhanced photodynamic therapy using angiostatin with verteporfin PDT in a laser-injury rat model. Invest Ophthalmol Vis Sci 44:1749
15. Takehana Y, Kurokawa T, Kitamura T, Tsukahara Y, Akahane S, Kitazawa M, Yoshimura N (1999) Suppression of laser-induced choroidal neovascularization by oral tranilast in the rat. Invest Ophthalmol Vis Sci 40:459–466
16. Berkowitz BA, Lukaszew RA, Mullins CM, Penn JS (1998) Impaired hyaloidal circulation function and uncoordinated ocular growth patterns in experimental retinopathy of prematurity. Invest Ophthalmol Vis Sci 39:391–397
17. Hughes A (1979) A schematic eye for the rat. Vision Res 19:569–588
18. Bikfalvi A, Sauzeau C, Moukadiri H, Maclouf J, Busso N, Bryckaert M, Plouet J, Tobelem G (1991) Interaction of vasculotropin/vascular endothelial cell growth factor with human umbilical vein endothelial cells: binding, internalization, degradation, and biological effects. J Cell Physiol 149:50–59
19. Vaisman N, Gospodarowicz D, Neufeld G (1990) Characterization of the receptors for vascular endothelial growth factor. J Biol Chem 265:19461–19466
20. Plouet J, Moukadiri H (1990) Characterization of the receptor to vasculotropin on bovine adrenal cortex-derived capillary endothelial cells. J Biol Chem 265:22071–22074
21. Kwak N, Okamoto N, Wood JM, Campochiaro PA (2000) VEGF is major stimulator in model of choroidal neovascularization. Invest Ophthalmol Vis Sci 41:3158–3164
22. Kim KJ, Li B, Houck K, Winer J, Ferrara N (1992) The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors 7:53–64
23. Fuh G, Wu P, Liang WC, Ultsch M, Lee CV, Moffat B, Wiesmann C (2006) Structure-function studies of two synthetic anti-vascular endothelial growth factor Fabs and comparison with the Avastin Fab. J Biol Chem 281:6625–6631
24. Campochiaro PA, The First ARVO/Pfizer Institute Working Group (2006) Ocular versus extraocular neovascularization: mirror images or vague resemblances. Invest Ophthalmol Vis Sci 47:462–474