Development of an Experimental Model of Proliferative Retinopathy by Intravitreal Injection of VEGF165

Emmerson Badaro, Eduardo Amorim Novais, Kalil Abdala, Mikael Chun, Muller Urias, Paulo Augusto de Arruda Melo Filho, Michel Eid Farah, and Eduardo Buchele Rodrigues

Abstract

Purpose: To develop an experimental model of proliferative retinopathy by intravitreal injection of vascular endothelial growth factor 165 (VEGF165) in pigmented rabbits.

Methods: A prospective, controlled, comparative intervention study. Six pigmented rabbits (Chinchilla breed) were subjected to intravitreal injection of VEGF165 in their right eye. The left eye was used as control and received an injection of balanced salt solution. In group 1, 3 rabbits received a 10- μ g injection, and in group 2, 3 rabbits received a 20- μ g injection. At baseline, all subjects were analyzed by anterior biomicroscopy, retinography, fluorescein angiography, and optical coherence tomography (OCT) fundus images. Biomicroscopy and all ancillary examinations were repeated at weeks 1, 2, and 5. In the fifth week after the injection, the rabbits were euthanized and the eyes were enucleated and subjected to histological evaluation.

Results: Seven days after the intravitreal VEGF165 injection, all rabbits developed intense neovascularization of the retina and anterior segment. Neovascularization of the posterior pole was similar in both groups, and the anterior segment was more florid in group 2. At weeks 1 and 2, neovascularization persisted with a minor decrease in conjunctival hyperemia in both groups. At week 5, there was a partial regression of neovascularization of the posterior pole, which was more prominent in group 1 than group 2, with persistent anterior neovascularization in both groups. OCT showed a statistically significant increase in retinal thickness, hyaloid detachment, and tractional retinal detachment. After the 5-week period, ocular histopathological evaluation showed an increase in retinal thickness, hyaloid detachment, and intense neovascularization in both groups, especially group 2.

Conclusion: This pilot study of a neovascularization model using intravitreal injection of VEGF165 in pigmented rabbits showed that both doses of 10 and 20 μ g were successful and effective in inducing vascular growth in the retina and anterior segment and can therefore be used for evaluating drug efficacy in future studies.

Introduction

SOME OF THE MOST important causes of blindness in the world are related to the phenomenon of intraocular neovascularization. Among these are wet age-related macular degeneration (AMD), diabetic retinopathy (DR), retinal vascular occlusions, and retinopathy of prematurity (ROP). AMD is the main cause of vision threat in the elderly population,¹ DR in the working age group,² and ROP in newborns,³ and the prevalence of these diseases tends to increase due to either increased life expectancy or better care for newborns. One common physiopathology of these diseases is ocular ischemia, which leads to high levels of intraocular vascular endothelial growth factor (VEGF) and secondary retinal or subretinal neovascularization.

VEGF is a dimeric protein known for its great potential to stimulate the growth of endothelial cells, to induce normal and pathological neovascularization, and to increase the permeability of capillary vessels.⁴ With advances in biotechnology, the isoform most commonly used in experimental studies to evaluate the angiogenic activity of VEGF is VEGF165.⁵

The development and progression of retinal neovascularization has been shown to be very similar both in humans and animal models currently used.⁶ Various experimental models of ocular neovascularization have been created to improve our knowledge of pathological angiogenesis and to optimize its treatment. Ischemia plays a central role in the development of retinal neovascularization, and animal models attempt to reflect it. The induction of proliferative retinopathy by intraocular

injection of proangiogenic molecules in rabbits and pigs^{6,7} is the best model for the study of drug action and better correlations with the anatomy and pathology of the human eye.

Each currently available model has advantages and disadvantages. Smaller animals have advantages such as lower maintenance costs, easier handling, and possibility of genetic alterations, but the large anatomic differences compared to humans make it difficult to extrapolate findings. Large animals, although more similar to humans, facilitating the analysis and correlation of results, have problems with high maintenance costs, dependence on skilled labor for handling, and ethical complications regarding their use in research.⁶⁻⁸

The goal of the current study was to establish, using an intravitreal injection of VEGF165, a sustainable and reproducible model of retinal neovascularization that is well documented with retinography, fluorescein angiography (FA), optical coherence tomography (OCT), and histological findings.

Methods

Six male Dutch-belted rabbits, weighing from 1.8 to 2.5 kg, were used in accordance with the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986), and the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of Animals in Ophthalmic and Vision Research, as well as university and national guidelines for research with animals.

The rabbits were anesthetized by intramuscular injection of ketamine hydrochloride (25 mg/kg) (Fort Dodge Animal Health, Fort Dodge, IA), and the pupils were dilated with 2.5% phenylephrine hydrochloride (Akorn, Inc., Buffalo Grove, IL) and 0.5% tropicamide (Akorn, Inc.) eye drops. Topical tetracaine (Bausch & Lomb, Inc., Tampa, FL) was used for additional anesthesia.

The animals were given an injection of 0.05 mL of 10 or 20 µg VEGF165 (BioLegend, San Diego, CA) into the right eye (OD) vitreous cavity, while injection of 0.05 mL of balanced salt solution (BSS) (290 mOsm) into the left eye (OS)

served as the control. These concentrations were chosen according to previous studies showing a correlation with proliferation of retinal vessels.⁷ After the placement of a lid speculum, a drop of topical 5% povidone-iodine was instilled followed by BSS washout. The vitreous cavity was accessed through the superotemporal sclera 2 mm posterior to the limbus, using a 27-gauge needle connected to a 1-mL syringe containing VEGF165 or BSS. Eyes were immediately examined by indirect ophthalmoscopy; 1 drop of antibiotic-steroid (0.5% moxifloxacin-0.1% dexamethasone) solution was then instilled in each eye.

Animals were divided into 2 groups: group 1 consisted of 3 rabbits that received an injection of 10 µg and group 2 consisted of 3 rabbits that received an injection of 20 µg. At baseline, all animals were subjected to slit-lamp examination, fundus photography, FA (Heidelberg Retina Angiograph 2; Heidelberg Engineering, Heidelberg, Germany), and spectral domain OCT (Spectralis; Heidelberg Engineering). All auxiliary examinations and biomicroscopy were repeated at weeks 1, 2, and 5. Before examination, the pupils were dilated and the rabbits were anesthetized as previously described. In the fifth week after injection, the rabbits were sacrificed and the eyes were enucleated and subjected to histological evaluation.

For FA, a slow (10–20 s) intravenous injection of 0.3 mL of 10% sodium fluorescein (Ophthalmos, Sao Paulo, Brazil) into the marginal ear vein was performed, and photographs were taken with the Heidelberg Retinal Angiography 2 within 30 s, 1 min, and 5 min after injection. Retinal thickness was measured using OCT in the vascular arcade 3 mm from the optic nerve and intergroup values were compared.

The rabbits were euthanized after 5 weeks of follow-up with an intravenous injection of 120 mg/kg sodium pentobarbital and their eyes were enucleated. All eyes were sectioned in half and fixed at 4°C in a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde in a 0.1 M phosphate buffer, pH 7.4. The specimens were stained en bloc with lead citrate, washed 3 times in a 0.1 M phosphate buffer, and dehydrated with ethyl alcohol. The specimens were embedded and stained with hematoxylin and eosin, and examined with an Optiphot-2TM light microscope (Nikon, Tokyo, Japan). Samples were obtained from 2 different areas

FIG. 1. Fundus pictures, FA, and optical coherence tomography (OCT) images of control group during the 5-week follow-up period. Normal findings from baseline until the end of the follow-up period. FA, fluorescein angiography.

FIG. 2. Fundus pictures, FA, and OCT images of group 1 during the 5-week follow-up period. Intense neovascularization and tortuosity of the vessels are notable in fundus and FA images at week 1, with normal OCT. From week 2 to 5, neovascularization and tortuosity decrease, but are still present on fundus and FA. OCT shows an increase of retinal thickness, hyaloid detachment, and on week 5, shows tractional retinal detachment.

in all dye-injected eyes in 3 serial sections: 500 μ m inferior to the optic nerve and 4 mm from the optic nerve in the inferior temporal quadrant. A horizontal diameter of the retinal surface of 1,100 μ m was used for detailed analysis of retinal toxicity.

The ocular examination was analyzed according to the anatomy of retinal vessels and evaluated for disc hyperemia, vascular dilation and tortuosity, or vitreous leakage of retinal neovascular membrane, vascular narrowing, pale disc, abnormal vascular pattern, and distorted and retinal elevation or noncapillary perfusion.

Results

Examinations of the rabbits in groups 1 and 2 showed several changes. One week after the injection of VEGF165, biomicroscopy showed that all rabbits had developed intense neovascularization of the retina and anterior segment. Neovascularization of the posterior pole was similar in both groups, but more florid anterior segment neovascularization was found in group 2. Neovascularization growth increased until week 2 and then slowly decreased until the end of follow-up. This decrease was more evident in group 1.

The control group showed no alterations on fundoscopy, FA, and OCT during the follow-up period (Fig. 1). FA in both groups showed leakage of the vessels in the retina 1

week after VEGF165 injection. In group 1, after week 1, this tissue slowly decreased until it was barely detectable by the end of the follow-up period (Fig. 2). However, capillary tortuosity persisted even after complete regression of neovascularization. In group 2, FA showed maximal leakage and neovascular changes at week 1 and persistence of the pattern until week 2, with a later decrease in the leakage pattern but continued capillary tortuosity (Fig. 3).

OCT showed a significant increase in average retinal thickness in groups 1 and 2 at week 1 of follow-up, after which, the thickness began to decrease. There was no significant ($P < 0.001$) difference between the groups at week 1, but average retinal thickness was significantly greater in group 2 (182.58 μ m) than in group 1 (173.50 μ m) at week 2 ($P < 0.001$) (Figs. 2 and 3). OCT also showed detachment of the posterior hyaloid in both groups. The OCT findings were similar for the groups by week 2 of the follow-up period, indicating areas of vitreoretinal traction close to the optic disc. Tractional detachments at the retinal medullary wings also developed by week 2 in group 2 and by week 5 in group 1.

Histopathologic examination of the groups revealed important differences between groups 1 and 2 and the control group. Groups 1 and 2 showed an increase in retinal thickness and the presence of neovascularization in the retina (Fig. 4). Group 2 had an increased number of vessels even at the end of follow-up.

FIG. 3. Fundus pictures, FA, and OCT images of group 2 during the 5-week follow-up period. Intense neovascularization and tortuosity of the vessels are notable in fundus and FA images at week 1 and 2 and decrease of these findings until week 5. At week 1, OCT shows an increase of retinal thickness, hyaloid detachment, and tractional retinal detachment increasing until the end of the follow-up period.

FIG. 4. Histology with hematoxylin-eosin light microscopy 5 weeks after intravitreal injection of VEGF in group 1 (left) and group 2 (right) showing hyaloid detachment, increase of retinal thickness, and the presence of neovascularization along on retina surface with red blood cell inside. VEGF, vascular endothelial growth factor.

Follow-up examinations of the rabbits in the control group (OS) showed no changes compared to baseline, indicating that intravitreal injection of BSS did not disrupt the blood–retina barrier or cause an inflammatory reaction.

Discussion

VEGF has been implicated as a major factor in the angiogenesis and progression of neovascular eye diseases, such as DR, AMD, vascular occlusion, and ROP. Among its effects in the eye, VEGF promotes the growth of vascular endothelial cells and increases vascular permeability. It plays a key role in embryonic vasculogenesis, including the development of choroidal vasculature, and is an important mediator in tumor angiogenesis.⁹ Complement activation and inflammation, with subsequent vascular damage, is thought to play a role in the upregulation of VEGF in these diseases.¹⁰ Due to its importance in the physiopathology of these diseases, one concept that has emerged from studies in the eye is that VEGF is an extremely good target for treatment of neovascular diseases.¹¹

In our study, we used VEGF165 because it is the most important factor related to pathological ocular neovascularization in several ocular diseases, and we believe that this model would be useful for testing antineovascularization drugs. For studies interested in biological mechanisms, transgenic and knockout models are useful models of neovascularization. For those interested in drug development, animal models that quickly develop neovascularization, such as drug- or laser-induced models, will continue to be important, especially since these animal models usually develop neovascularization in days to weeks as opposed to the usual months to years it takes for transgenic and knockdown models.⁶ As new drugs have developed and become successful, it will become increasingly more difficult to prove superiority of one drug versus another. However, to do this, animal models will continue to be needed to test for safety and efficacy. New reproducible animal models will continue to be developed, especially larger animals for drug delivery studies.

The current study showed that the injection of 10 or 20 μ g of VEGF165 into the vitreous cavity of rabbit eyes caused retinal changes that led to fluorescein leakage and neovascularization. Because leakage from the anomalous vessels was detected after 7 days, we believe that this may be the best time to test new drugs that can help prevent and reverse this process. The dose of 10 μ g may be sufficient to achieve and maintain retinal neovascularization, since marked leakage was observed in the first days after intravitreal injection.

Since the retina of rabbits is essentially avascular, except for the medullary rays, one should be aware that neovascularization is only found in the area of the original vessels. This creates a good method for measuring the area of neovascularization more precisely and following its regression with more accuracy as the assessment of the peripheral retina is not necessary.

The advantages of our study were that animals showed acceptable levels of neovascularization during the follow-up period. Other methods available in literature are intravitreal injection of fibroblasts,¹² transgenic animals,¹³ and subretinal injection of recombinant adeno-associated virus-mediated VEGF.¹⁴ Also, we could identify neovessels in a short time after the injection and there was a long follow-up period (5 weeks). Limitations were the small number of animals, doses, and regimens of injections tested, and the absence of measures of VEGF165 levels in the vitreous cavity.

Among the neovascular retinal diseases, some can be pointed out as benefiting from this model, such as DR, retinal vascular occlusions, and ROP. Some innovative image modalities, such as OCT angiography and swept-source OCT, may be useful in assessing this model and may be used in future studies.

In conclusion, this pilot study of the model of retinal neovascularization using intravitreal VEGF165 injection in pigmented rabbits showed that both doses of 10 and 20 μ g were successful and effective in inducing retinal neovascularization and, hence, can be used for evaluating the efficacy of drugs in future studies. The current study described a simple, efficient, sustainable, reliable, and reproducible animal model, which was well documented by FA, OCT, and histopathological analysis.

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Author Disclosure Statement

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Address correspondence to:

Dr. Emmerson Badaro

Department of Ophthalmology

Vision Institute (IPEPO)

Federal University of São Paulo

Rua Botucatu 821, 1st floor

São Paulo/SP, 04023-062

Brazil

E-mail: ebadaro@me.com