AAV8-antiVEGFfab Ocular Gene Transfer for Neovascular Age-Related Macular Degeneration

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Sustained suppression of VEGF is needed in many patients with neovascular age-related macular degeneration (NVAMD), and gene transfer of a VEGF-neutralizing protein is a promising approach to achieve it. Initial clinical trials testing this approach have shown encouraging signals, but evidence of robust transgene expression and consistent antiangiogenic and antipermeability activity has been lacking. In this study, we demonstrate expression of an anti-human VEGF antibody fragment (antiVEGFfab) after subretinal injection of AAV8-antiVEGFfab. In transgenic mice expressing human VEGF in retina (rho/VEGF mice), a model of type 3 choroidal neovascularization (NV), eyes injected with $\geq 1 \times 10^7$ gene copies (GC) of AAV8-antiVEGFfab had significantly less mean area of NV than null vector-injected eyes. A dose-dependent response was observed with modest reduction of NV with $\leq 3 \times 10^7$, >50% reduction with $\geq 1 \times 10^8$ GC and almost complete elimination of NV with 3×10^9 or 1×10^{10} GC. In Tet/opsin/VEGF mice, in which doxycycline-induced high expression of VEGF leads to severe vascular leakage and exudative retinal detachment (RD), reduction of total RD by 70%–80% occurred with 3×10^9 or 1×10^{10} GC of AAV8-antiVEGFfab, an effect that was sustained for at least a month. These data strongly support initiating clinical trials testing subretinal injection of AAV8-antiVEGFfab in patients with NVAMD.

INTRODUCTION

Age-related macular degeneration (AMD) is a highly prevalent neurodegenerative disease in which death of photoreceptors and retinal pigmented epithelial (RPE) cells results in gradual loss of central vision. A subgroup of 10%–15% of patients with AMD develop subretinal neovascularization (NV) resulting in relatively rapid reduction in visual acuity due to leakage of plasma from incompetent new vessels and collection of fluid within and under the retina, which compromises retinal function. This subgroup is said to have neovascular AMD (NVAMD).

Vascular endothelial growth factor (VEGF) plays a central role in the development of subretinal NV and excessive leakage from the NV causing subretinal and/or intraretinal fluid in the macula that decreases vision. Intraocular injections of VEGF-neutralizing proteins reduce leakage, allowing fluid reabsorption and improvement in visual acuity;[1,2] however, the production of VEGF is chronic, so that leakage and NV growth recur when vitreous levels of a VEGF-neutralizing protein drop below therapeutic levels. In initial clinical trials, treatment-naive subjects with recent onset of NVAMD were given monthly intravitreous injections of a VEGF-neutralizing protein, and 34%–40% of subjects experienced at least a 15-letter improvement in best-corrected visual acuity (BCVA), a large and clinically meaningful benefit, that was maintained for 2 years.[1,2] In an extension study in which subjects were seen and treated as infrequently as every 3 months, almost all of the visual benefits were lost.[3] In a subsequent study in subjects with NVAMD, the mean improvement from baseline BCVA was significantly greater in those given monthly injections of a VEGF-neutralizing protein compared to those who had monthly visits with injections only when intraretinal or subretinal fluid was present in the macula.[4] Visual benefits were maintained for 2 years with these treatment regimens, after which subjects were treated at the discretion of their physician, and after 3 years, visual benefits were lost and mean BCVA in all groups was worse than baseline.[5] Thus, frequent injections with sustained suppression of VEGF for patients with NVAMD, may be one of several factors needed to maximize and maintain visual benefits.

One strategy to provide long-term benefits in patients with NVAMD is ocular gene transfer to continuously express an antiangiogenic

Figure 1. Schematic of AAV8-antiVEGFfab Genome and Transgene Expression after Subretinal Injection in Mice

(A) Schematic of AAV8-antiVEGFfab genome. (B) Adult C57BL/6 mice were given a subretinal injection of 10^{10} genome copies (GC) of empty AAV8 vector or a dose of AAV8-antiVEGFfab between 1×10^{8} and 1×10^{10} GC. After 7 days, mice were euthanized and eyes were removed and frozen until assayed. Eyes were homogenized in lysis buffer, and AAV8-antiVEGFfab protein was measured by ELISA. Bars represent the mean (±SEM) with n = 10 for each bar.

protein within the retina. This approach strongly suppresses retinal or subretinal NV in animal models.[6–14] In subjects with advanced NVAMD, intravitreous injection of an adenoviral vector expressing pigment epithelium-derived factor caused reabsorption of subretinal hemorrhage and fluid in several patients with advanced NVAMD, providing proof of concept for this approach.[15] AAV vectors are an appealing platform because they provide long-term transgene expression. A recent phase 1 clinical trial in subjects with advanced NVAMD tested intravitreous injection of an AAV2 vector containing a chicken beta actin (CBA) promoter driving expression of a VEGF-neutralizing protein consisting of domain 2 of Flt-1 (VEGFR1) linked by a polyglycine 9-mer to human IgG1-Fc (sFLT01).[16] Transgene expression was detected in 5 of 10 eyes injected with the highest dose, 2×10^{10} genome copies (GC), and none of the eyes with $<2 \times 10^{10}$ GC. In 11 of 19 patients with intraretinal or subretinal fluid at baseline judged to be reversible, six showed substantial fluid reduction and improvement in vision, whereas five showed no fluid reduction. Thus, while there was some evidence of biologic activity, there was considerable heterogeneity among patients with regard to response. In general, compared with intravitreous injections of
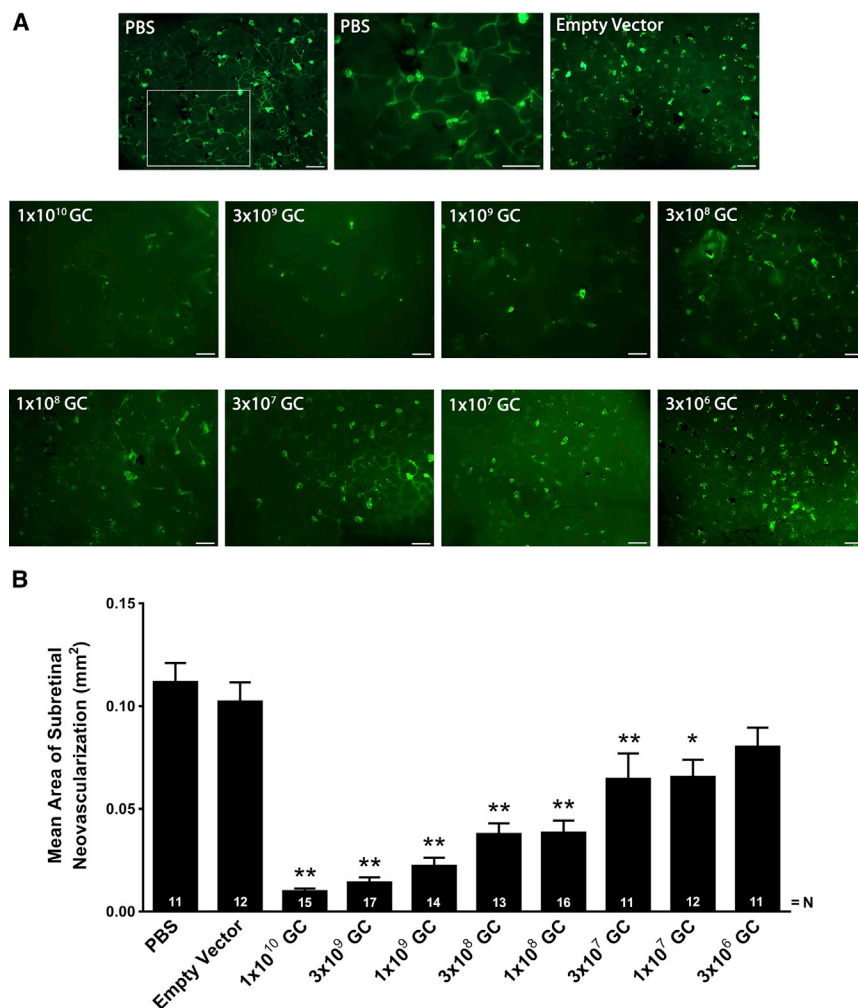
AAV2 vectors, subretinal injections provide substantially higher transgene expression, and a phase 1 trial testing subretinal injection of an AAV2 vector in which the *cytomegalovirus* (*CMV*) promoter drives expression of native soluble VEGFR1 (AAV2-sFlt-1) in subjects with NVAMD showed good safety, and 3 of 6 subjects given a subretinal injection of 1×10^{10} or 1×10^{11} GC of AAV2.sFlt-1 showed some reduction of intraretinal fluid.[17] In a phase 2 trial of 32 subjects with NVAMD, 21 were randomized to subretinal injection of 100 μL containing 1×10^{11} GC of AAV2.sFlt-1, and 11 were randomized to ranibizumab injections only as needed for recurrent intraretinal or subretinal fluid.[18] All subjects received ranibizumab injections at baseline and week 4 and thereafter according to prespecified criteria. The study failed to demonstrate sufficient efficacy to continue development of AAV2.sFlt-1. Levels of sFlt-1 were not reported and therefore lack of sufficient sFlt-1 expression cannot be ruled out as a potential factor in the lack of response seen in the study. These prior studies suggest improvements in vectors, expression cassettes, anti-VEGF protein, injection techniques, or some combination of these strategies are needed to improve transgene expression for gene transfer to effectively treat NVAMD.

Screening of tissues from rhesus monkeys by PCR for sequence homologies to known AAV serotypes led to the identification of AAV7 and AAV8.[19] Vectors with AAV8 capsids were generated, and in liver, transgene expression was 10- to 100-fold higher than with AAV vectors with a capsid of another serotype such as AAV2. Neutralizing antibodies to AAV8 were rare in human serum and antibodies to other AAV serotypes did not reduce AAV8 vector-mediated expression.[19] AAV2 and AAV8 both transduce RPE cells after subretinal injection of moderate or low doses, but AAV8 is more efficient at transducing photoreceptors and therefore results in overall higher expression levels.[20] Utilization of AAV8 vectors to express a VEGF-neutralizing protein may help to maximize long-term suppression of VEGF in the retina and overcome shortcomings of past gene transfer approaches in NVAMD. In this study, we utilized transgenic mouse models in which human $VEGF_{165}$ is expressed in photoreceptors to test the efficacy of subretinal injection of a wide range of doses of an AAV8 vector containing an expression cassette for a humanized antibody fragment that binds human VEGF.

RESULTS

Construction and *In Vitro* Testing of AAV8-antiVEGFfab

The cDNA for antiVEGFfab was inserted into an expression cassette containing a CB7 promoter. A schematic of the genome of AAV8-antiVEGFfab (RGX-314) is shown in Figure 1A. The CB7 promoter drives expression of the heavy and light chain of antiVEGFfab and a Furin-F2A linker resulting in post-translational assembly of antiVEGFfab. The light chain is preceded by a signal peptide that is cleaved, ensuring that no additional amino acids are added to the light chain. Heavy-chain processing relies on endogenous carboxypeptidases to cleave positively charged residues remaining after furin cleavage, and there may be some heterogeneity at the C terminus that would not affect dimer formation or anti-VEGF activity.[21]

A

B

escent spots (Figure 2A, top row, left column). At higher magnification, feeder vessels are seen extending from the deep capillary bed in the background to the buds of subretinal NV partially surrounded by dark black RPE cells (Figure 2A, top row, middle column). Mice injected with 1×10^{10} GC of empty AAV8 vector at P14 showed comparable amounts of subretinal NV at P21 as that seen in PBS-injected eyes (Figure 2A, top row, right column). *Rho/VEGF* mice given a subretinal injection of 1×10^{10}, 3×10^{9}, or 1×10^{9} GC of AAV8-antiVEGFfab showed very little subretinal NV at P21 (Figure 2A, middle row), while those injected with 3×10^{8} or 1×10^{8} GC (Figure 2A, middle and bottom rows) showed somewhat more, but still considerably less than mice injected with empty vector. An intermediate amount of NV was seen in mice injected with 3×10^{7} and 1×10^{7} GC, and those injected with 3×10^{6} GC appeared similar to those injected with empty vector (Figure 2A, bottom row). Measurement of the mean area of NV per retina by image analysis showed a dose response that paralleled what visual inspection of the retinal flat mounts suggested, with mean area of NV per retina significantly less in eyes injected with doses of AAV8-antiVEGFfab between 1×10^{10} and 1×10^{7} GC than in empty vector-injected eyes (Figure 2B).

Transgene Expression after Subretinal Injection of AAV8-antiVEGFfab in Mice

One week after subretinal injection of 1 μL of doses ranging from 1×10^{8} to 1×10^{10} GC of AAV8-antiVEGFfab, the level of anti-VEGFfab protein was measured in eye homogenates. As vector dose was increased, there was an increase in antiVEGFfab with peak levels obtained at doses of 3×10^{9} and 1×10^{10} GC (Figure 1B).
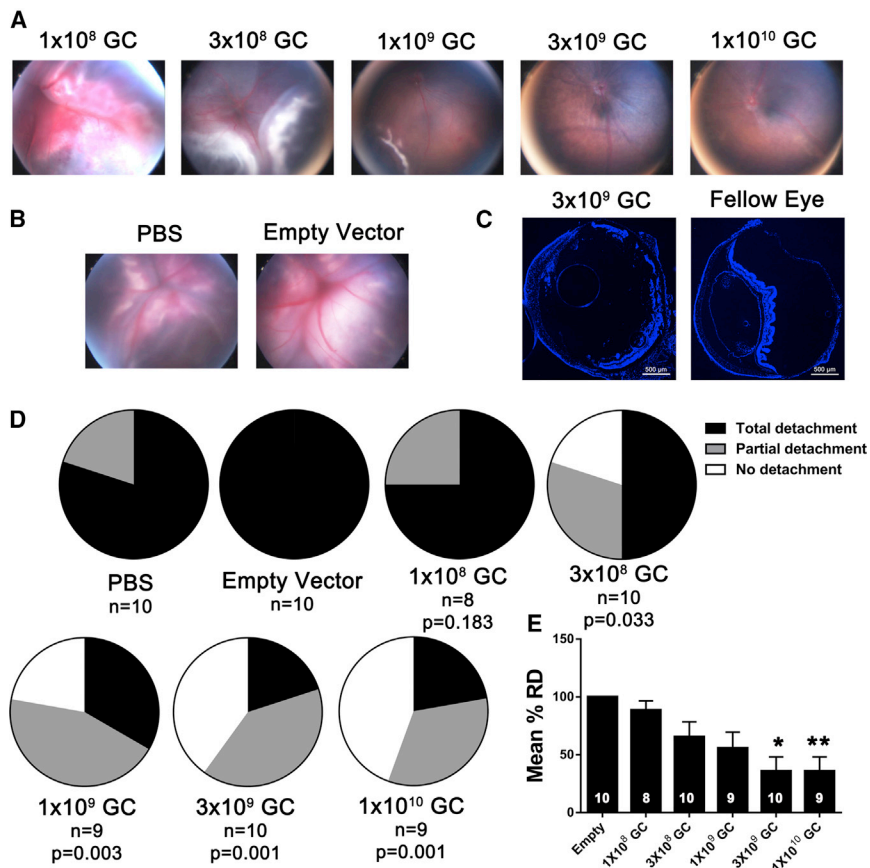
Subretinal Injection of AAV8-antiVEGFfab Suppresses Subretinal NV in *rho/VEGF* Mice

Transgenic mice in which the *rhodopsin* promoter drives expression of human VEGF$_{165}$ sprout new vessels from the deep capillary bed starting around postnatal day (P) 14 and have extensive subretinal NV by P21.[22,23] These mice provide a model for retinal angiomatous proliferation, also known as type 3 choroidal NV in humans.[24] After subretinal injection of PBS at P14, retinas of rho/VEGF mice stained with fluorescein isothiocyanate (FITC)-labeled *Griffonia simplicifolia* (GSA) lectin, which selectively stains vascular cells, were flat mounted with the photoreceptor side facing upward and showed numerous hyperfluor-

Subretinal Injection of AAV8-antiVEGFfab Blocks VEGF-Induced Vascular Leakage

Tet/opsin/VEGF double transgenic mice, in which the tet-on system and the *rhodopsin* promoter provide doxycycline-inducible
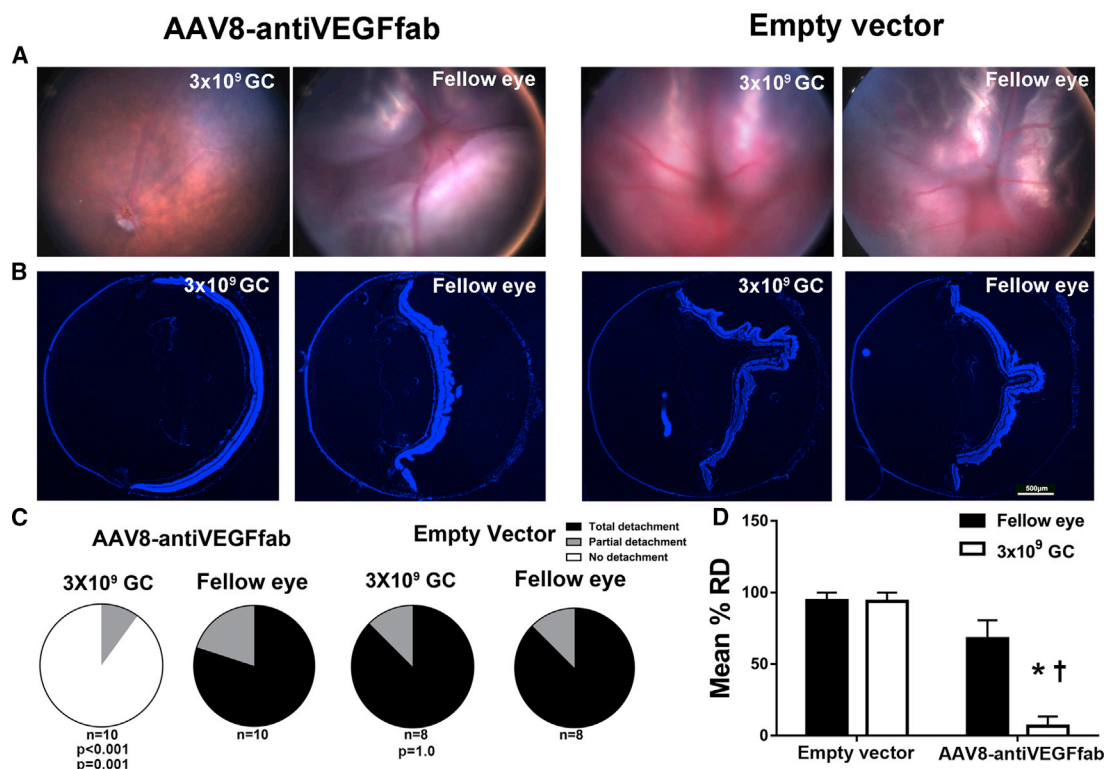
Figure 3. Prevention of Severe VEGF-Induced Leakage and Exudative Retinal Detachment by Subretinal Injection of AAV8-antiVEGFfab

Adult *Tet/opsin/VEGF* double transgenic mice had a subretinal injection of AAV8-antiVEGFfab in doses ranging from 1×10^8 to 1×10^{10} GC in one eye and no injection in the fellow eye or 1×10^{10} GC of null vector in one eye and PBS in the fellow eye. Ten days after injection, 2 mg/mL of doxycycline was added to drinking water and after 4 days fundus photos were graded for presence of total, partial, or no retinal detachment, and the percentage of detachment was measured for each eye. Representative fundus photos show total retinal detachments in mice injected with 1×10^8 or 3×10^9 GC of AAV8-antiVEGFfab (A, left) similar to those seen in mice injected with PBS or empty vector (B). There was a partial retinal detachment in an eye injected with 1×10^9 GC and no retinal detachment in eyes injected with 3×10^9 or 1×10^{10} GC of AAV8-antiVEGFfab (A, right three columns). An ocular section stained with Hoechst from an eye injected with 3×10^9 GC of AAV8-antiVEGFfab shows no retinal detachment, while a section from the uninjected fellow eye shows a total retinal detachment (C). Pie charts show a dose-dependent reduction in exudative retinal detachments in eyes injected with AAV8-antiVEGFfab (D). p values performed by Fisher's test show for different doses whether there is a difference regarding the presence of no, partial, or total detachment from the empty vector group. (E) The mean (±SEM) percentage retinal detachment was significantly less for eyes injected with 3×10^9 or 1×10^{10} GC of AAV8-antiVEGFfab than for any of the other groups by one-way ANOVA with Bonfferoni correction for multiple comparisons (*p = 0.002, **p = 0.001).

expression of $VEGF_{165}$ at levels 30-fold higher than those present in the retinas of *rho/VEGF* mice, develop exudative retinal detachment within 4 days of starting 2 mg/mL doxycycline in drinking water.[25] Ten days after subretinal injection of doses of AAV8-antiVEGFfab ranging from 1×10^8 to 1×10^{10} GC of AAV8-antiVEGFfab, 1×10^{10} GC of empty vector, or PBS in *Tet/opsin/VEGF* mice, 2 mg/mL of doxycycline was added to their drinking water. Representative fundus photographs of mice from each group are shown in Figures 3A and 3B. Most mice in the 1×10^8 and 3×10^8 GC dose groups had total exudative retinal detachment (Figure 3A, left two panels), similar to what was seen in the PBS and empty vector groups (Figure 3B). In contrast, the most common finding in the 1×10^9 GC group was partial retinal detachment (Figure 3A, middle panel), and in the 3×10^{10} and 1×10^{10} GC groups, many mice had no retinal detachment (Figure 3A, right two panels). Figure 3C shows an ocular section from an eye injected with 3×10^9 GC of AAV8-anti-VEGFfab showing no exudative retinal detachment and an uninjected fellow eye with a total retinal detachment. Four days after starting doxycycline, 10 of 10 eyes given a subretinal injection of empty vector and 8 of 10 injected with PBS had total retinal detachment, and 10 of 10 eyes given a subretinal injection of 1×10^{10} GC of empty vector had total (7 eyes) or partial (3 eyes) retinal detachment (Figure 3D). Compared with mice injected with empty vector, the percentage of

eyes with total retinal detachment was significantly less in doxycycline-treated *Tet/opsin/VEGF* mice that had been given a subretinal injection of 3×10^8 (50% less), 1×10^9 (67% less), 3×10^9 (80% less), or 1×10^{10} (78% less) GC of AAV8-antiVEGFfab. The percentage of the retina that was detached was measured in each eye by image analysis by masked investigators and compared with eyes injected with empty vector; the mean percentage detachment was significantly less in eyes injected with 3×10^9 or 1×10^{10} GC of AAV8-antiVEGFfab (Figure 3E).

To assess long-term effects, Tet/opsin/VEGF mice were treated with 2 mg/mL doxycycline 1 month after subretinal injection of 3×10^9 GC of AAV8-antiVEGFfab or empty vector in one eye. Representative fundus photographs from each group in Figure 4A mice show no detachment in the AAV8-antiVEGFfab-injected eye and total detachment in the fellow eye (Figure 4A, left side), and total detachment in both the empty-vector-injected eye and fellow eye (Figure 4A, right side). In other mice from the two groups, Hoecht-stained ocular sections show no detachment in an AAV8-antiVEGFfab-injected eye and total detachment in the fellow eye, and total detachment in both an empty vector-injected eye and fellow eye (Figure 4B). Nine of ten eyes injected with AAV8-antiVEGFfab had no retinal detachment, which was significantly different from uninjected fellow eyes in the

Figure 4. Subretinal Injection of AAV8-AntiVEGFfab Prevents Doxycycline-Induced Exudative Retinal Detachment for at Least 1 Month

Adult *Tet/opsin/VEGF* double transgenic mice had subretinal injection of 3×10^9 GC AAV8-antiVEGFfab in one eye and no injection in the fellow eye or 3×10^9 GC of null vector in one eye and no injection in the fellow eye. One month after injection, 2 mg/mL of doxycycline was added to drinking water, and after 4 days fundus, photos were graded for presence of total, partial, or no retinal detachment, and the percentage of the retina detached was measured in each eye. Fundus photos from a representative mouse injected with 3×10^9 GC of AAV8-antiVEGFfab show no detachment in the injected eye and total detachment in the fellow eye (A, left two panels), while those from representative mouse injected with 3×10^9 GC empty vector show severe total retinal detachment in each eye (A, right two panels). Ocular sections stained with Hoechst from a mouse injected with 3×10^9 GC of AAV8-antiVEGFfab show attached retina in the injected eye and total detachment in the fellow eye (B, left two panels). Ocular sections from a mouse injected with 3×10^9 GC empty vector show total retinal detachment in each eye (B, left two panels). (C) Nine of ten eyes injected with AAV8-antiVEGFfab had no retinal detachment, while 8 of 10 fellow eyes had total retinal detachment (p < 0.001 by Fisher's test). In contrast, seven of eight eyes injected with empty vector and fellow eyes had total retinal detachment (p = 1.0). Compared to eyes injected with empty vector, there was significant prevention of retinal detachment in eyes injected with AAV8-antiVEGFfab (p = 0.001 by Fisher's test). (D) In eyes injected with AAV8-antiVEGFfab (n = 10) the mean (±SEM) percentage retinal detachment (RD) per eye was significantly less than that in fellow eyes (*p < 0.001) or eyes injected with empty vector (n = 8; †p = 0.001 by one-way ANOVA with Bonferroni correction).

same mice, for which eight eyes had total detachment and two eyes had partial detachment (Figure 4C). Compared with eight eyes injected with empty vector, in which there were seven total and one partial detachments, those injected with AAV8-antiVEGFfab showed significantly fewer detachments. Also, the mean percentage retinal detachment in eyes injected with AAV8-antiVEGFfab was significantly less than that in uninjected fellow eyes in the same mice or eyes injected with empty vector (Figure 4D).

DISCUSSION

Sustained suppression of VEGF is needed in most patients with NVAMD to maximally improve visual acuity and prevent disease progression and vision loss over time. Several strategies designed to achieve this goal are being tested. Surgical implantation of a refillable reservoir that slowly releases a VEGF-neutralizing protein into the eye is being evaluated in a phase 2 clinical trial in patients with

NVAMD (Study of the Efficacy and Safety of the Ranibizumab Port Delivery System for Sustained Delivery of Ranibizumab in Participants With Subfoveal Neovascular Age-Related Macular Degeneration; ClinicalTrials.gov identifier, NCT02510794). Another approach is to incorporate a small molecule inhibitor of HIF-1, which suppresses expression of VEGF, into a biodegradable polymer, formulate microparticles that allow sustained release of the inhibitor, and inject them into the eye.[26] A third approach is ocular gene transfer to express a VEGF-neutralizing protein or other antiangiogenic protein. Clinical trials testing the gene transfer approach in patients with NVAMD have shown encouraging signals[15–18] but have failed to show consistent, sustained transgene expression and/or strong anti-permeability and antiangiogenic activity.

In this study, we found that subretinal injection of 1×10^9 to 1×10^{10} GC of AAV8-antiVEGFfab strongly suppressed type 3

choroidal NV in *rho/VEGF* mice. The minimally effective subretinal dose of AAV8-antiVEGFfab, which significantly reduced the mean area of subretinal NV per retina compared with subretinal injection of empty vector was 1×10^7 GC. In *Tet/opsin/VEGF* double-transgenic mice with doxycycline-inducible expression of at least 30-fold higher doses of VEGF compared with that in rho/VEGF mice, 10 days after subretinal injection of AAV8-antiVEGFfab doses as low as 3×10^8 GC, there was significant reduction in the incidence and severity of exudative retinal detachments. Leakage suppression was particularly good 10 days after injection of 3×10^9 or 1×10^{10} GC, which showed a significant reduction in mean percentage retinal detachment. Most eyes injected with 1×10^8 GC or greater had detectable levels of antiVEGFfab with peak levels of 60–80 ng per eye after injection of 3×10^9 or 1×10^{10} GC. To assess effects at a longer time point, Tet/opsin/VEGF mice were given doxycycline 1 month after subretinal injection of 3×10^9 GC of AAV8-antiVEGFfab or empty vector, and results were dramatic, with no detachment in 9 of 10 eyes and one partial detachment in the AAV8-antiVEGFfab group, compared with total detachment in seven of eight eyes and one partial detachment in the empty vector group.

These data are encouraging and suggest that subretinal injection of AAV8-antiVEGFfab may help to overcome some of the problems encountered in prior gene transfer clinical trials for NVAMD. In the AAV2-sFLT01 study, aqueous humor samples were assayed for sFLT01, and all samples from subjects injected with 2×10^8, 2×10^9, or 6×10^9 GC were below the lower limit of detection at all time points, but 5 of 10 subjects injected with 2×10^{10} GC had detectable levels that peaked at 32.7 to 112.0 ng/mL (mean 73.7 ng/mL) by week 26 with a slight decrease to a mean of 53.2 ng/mL at week 52.[16] Pre-existent neutralizing antibodies have been found to neutralize intravitreal gene therapy in nonhuman primates,[27] and anti-AAV2 serum antibodies might explain this variability in expression in this trial. Four of the five subjects with detectable sFLT01 levels were negative for anti-AAV2 antibodies at baseline, and the fifth had a 1:100 titer, whereas four of the five high-dose subjects with undetectable sFLT01 levels had titers ≥ 1:400. The incidence of anti-AAV8 serum antibodies in the general population is far less than the incidence of anti-AAV2 antibodies.[28] In addition, subretinal injection of AAV8 is likely to provide an additional factor that may help protect from vector inactivation, because anti-AAV2 serum antibodies do not appear to prevent transgene expression after subretinal injections of AAV2 vectors.[27] Also, since transgene expression is substantially higher after subretinal versus intravitreous injection of AAV vectors and because at equivalent doses, transgene expression is greater with subretinal injection of AAV8 versus AAV2 vectors,[20] the approach taken in the current study may increase the chances of success in treatment of patients with NVAMD.

Some investigators have expressed concern that VEGF may provide trophic support to retinal neurons and that sustained suppression of VEGF could promote death of photoreceptors and other retinal neurons. In transgenic mice, induced, high level expression of a potent VEGF-neutralizing protein for up to 7 months caused no reduction in retinal function or evidence of death of photoreceptors or other retinal neurons.[29] Prolonged blockade of VEGF receptors with a small molecule tyrosine kinase inhibitor also caused no loss of retinal function or identifiable loss of photoreceptors or ganglion cells.[30] Studies investigating the safety of subretinal injections of AAV8-anitVEGFfab have been completed in nonhuman primates and along with the efficacy data reported herein have enabled an ongoing clinical trial to evaluate the safety and efficacy of AAV8-anti-VEGFfab in patients with NVAMD.

MATERIALS AND METHODS

Mice

All mice were treated in accordance with the Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research, and protocols were reviewed and approved by the Johns Hopkins University Animal Care and Use Committee. Transgenic mice in which the *rhodopsin* promoter drives expression of $VEGF_{165}$ in photoreceptors (*rho/VEGF* mice)[22,23] and double-transgenic mice with inducible expression of VEGF in photoreceptors (*Tet/opsin/VEGF* mice)[25] have been previously described. All transgenic mice were in C57BL/6 background and were genotyped to confirm the presence of transgenes prior to use in experiments. Wild-type C57BL/6 mice were purchased from Charles River (Frederick, MD, USA).

Subretinal Injections of Vector

Mice were anesthetized and eyes were visualized with a Zeiss Stereo Dissecting Microscope (Zeiss, Oberkochen, Germany). A 30-gauge needle on an insulin syringe was used to create a small partial thickness opening in the sclera and a 33G needle on a Hamilton syringe (Hamilton Company, Reno, NV) was inserted into the scleral puncture and slowly advanced through the remaining scleral fibers into the subretinal space, and 1 μL of vehicle-containing vector was injected. A cotton swab was applied to the injection site as the needle was removed to prevent reflux.

Construction of AAV8-antiVEGFfab

AAV8-antiVEGFfab is a non-replicating AAV8 vector containing a gene cassette encoding a humanized monoclonal antigen-binding fragment that binds and inhibits human VEGF, flanked by AAV2 inverted terminal repeats (ITRs). Expression of heavy and light chains is controlled by the CB7 promoter consisting of the chicken β-actin promoter and CMV enhancer, a chicken β-actin intron, and a rabbit β-globin poly(A) signal. The nucleic acid sequences coding for the heavy and light chains of antiVEGFfab are separated by a self-cleaving furin (F)/F2A linker. The expressed protein product is similar, but not identical, to ranibizumab. Due to the mechanism of furin-mediated cleavage, the vector-expressed antiVEGFfab may contain none, one, or more additional amino acid residues in the last position of the heavy chain in addition to all the amino acids normally found in ranibizumab.

Expression of antiVEGFfab Protein in the Retina

Wild-type C57BL/6 mice received a single subretinal injection in each eye of 1×10^8, 3×10^8, 1×10^9, 3×10^9, 1×10^{10} GC of

AAV8-antiVEGFfab, or 1×10^{10} GC of empty vector. Fourteen days after subretinal injection vector, the total amount of antiVEGFfab was measured by ELISA. Eyes were homogenized in 200 uL radioimmunoprecipitation assay (RIPA) buffer using QIAGEN Tissue Lyser (QIAGEN, Hilden, Germany). The concentration of antiVEGFfab protein was measured by ELISA using known concentrations of ranibizumab (Genentech, South San Francisco, CA) to generate a standard curve. In brief, ELISA plates were coated with 1 μg/mL of human $VEGF_{165}$ overnight at 4°C. Wells were blocked with 200 μL 1% BSA for 1 hr at room temperature. Samples were diluted 1:80, and 100 μL was added to duplicate wells, incubated for 1 hr at 37°C, and followed by a second blocking buffer incubation. After washing, wells were incubated for 1 hr at room temperature in 100 μL of a cocktail of 1 mg/mL goat anti-human immunoglobulin G (IgG) heavy chain and 0.5 mg/mL goat anti-human IgG light chain, both labeled with biotin and pre-absorbed (Abcam, Cambridge, MA). After washing, wells were incubated for 1 hr at room temperature in 100 μL of a 1:30,000 dilution of Streptavidin-HRP (Abcam, Cambridge, MA), washed, and incubated in 150 μL tetramethylbenzidine (TMB) detection solution consisting of 0.1 M NaOAc citrate buffer (pH 6.0), 30% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO), 3,3′,5,5′-tetramethylbenzidine ≥99% (Sigma-Aldrich, St. Louis, MO) at room temperature in the dark for 30 min, after which 50 μL of stop solution (2 N H_2SO_4) was added to each well, and the plate was read at 450–540 nm.

Assessment of Effects on Type 3 Choroidal NV in Rho/VEGF Mice

At P 14, rho/VEGF mice were given a single subretinal injection in one eye of 3×10^6, 1×10^7, 3×10^7, 1×10^8, 3×10^8, 1×10^9, 3×10^9, 1×10^{10} GC of AAV8-antiVEGFfab, or 1×10^{10} GC of empty vector or PBS. At P21, mice were euthanized, eyes were removed, and retinas were dissected intact, stained with FITC-conjugated GSA lectin (Vector Laboratories, Burlingame, CA), and flat-mounted with photoreceptor side facing up. Fluorescent images were obtained with a Zeiss Axioskop fluorescence microscope, and the area of type 3 choroidal NV per retina was measured by image analysis using ImagePro Plus software (Media Cybernetics, Rockville, MD) with the investigator masked with regard to treatment group.

Assessment of Effects on Severe VEGF-Induced Vascular Leakage

Ten-week-old *Tet/opsin/VEGF* mice were given a single subretinal injection in one eye of 1×10^8, 3×10^8, 1×10^9, 3×10^9, 1×10^{10} GC of AAV8-antiVEGFfab, or 1×10^{10} GC of empty vector or PBS. Ten days after injection, 2 mg/mL of doxycycline (Sigma-Aldrich, St. Louis, MO) was added to drinking water and after 4 days, mice were anesthetized, pupils dilated, and fundus photographs were obtained with a Micron III Retinal Imaging Microscope (Phoenix Research Laboratories, Pleasanton, CA). Images were examined by a masked investigator and were determined to show no, partial, or total exudative retinal detachment. The total retinal area and area of detached retina were measured by image analysis using ImagePro Plus software (Media Cybernetics, Rockville, MD) with the investi-

gator masked with regard to treatment group. The percentage retinal detachment was calculated as area of detached retina/total retina. In a small number of eyes, sharp fundus images could not be obtained due to lack of cornea or lens clarity, and in those cases, mice were euthanized, eyes were removed and frozen, and 10 μm serial sections were cut. Sections were post-fixed in 4% paraformaldehyde, stained with Hoechst (Vector Laboratories, Burlingame, CA) and examined by light microscopy to determine the presence and extent of exudative retinal detachment.

To investigate long-term effects, *Tet/opsin/VEGF* mice were given a single subretinal injection of 3×10^9 GC of AAV8-antiVEGFfab or 3×10^9 GC of empty vector in one eye. The fellow eye served as an untreated control. One month after injection, 2 mg/mL of doxycycline was added to drinking water, and after 4 days, fundus photographs were obtained and graded as described above.

Statistical Comparisons

Student's t tests were carried out to compare the outcome measures between two experiment groups. For comparisons among three or more experiment groups, one-way ANOVA adjusting for multiple comparison using Bonferroni multiple-comparison correction or mixed effects model accounting for the correlation between eyes from the same subject were performed. For comparing the types of detachment between the groups with different doses versus the empty vector group, the p values were calculated using the Fisher's exact tests. All statistical tests were conducted at 5% statistical significance. Statistical analyses were performed using Stata version 14.2 (College Station, Texas 77845).

AUTHOR CONTRIBUTIONS

Y.L., S.D.F., J.S., and J.W. performed efficacy studies in mouse models, analyzed data, performed statistical analyses, created figures, and contributed to writing the manuscript. E.W. and A.T. performed measurements of transgene expression and edited the manuscript. A.T. designed the optimized Fab vector and contributed to the design of the animal experiments. J.W. helped design animal experiments and supervised the design and construction of the vector, supervised measurements of transgene expression, and edited the manuscript. P.A.C. designed experiments, supervised efficacy studies, analyzed data, and wrote the first draft of manuscript. S.Y. and K.K. helped to design experiments and edited the manuscript.

CONFLICTS OF INTEREST

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