Aflibercept Action in a Rabbit Model of Chronic Retinal Neovascularization: Reversible Inhibition of Pathologic Leakage With Dose-Dependent Duration

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PURPOSE. We establish and characterize the chronic retinal neovascularization (RNV) induced by intravitreal (IVT) injection of DL-α-aminoadipic acid (AAA) in a rabbit model and investigate the extent and duration of inhibitory actions induced by IVT aflibercept on the RNV.

METHODS. Rabbits received a single IVT injection of AAA, with weekly follow-up fundus photography, fluorescein angiography (FA), and optical coherence tomography (OCT). After 10 weeks, they received a single IVT aflibercept or control injection. RNV leakage was quantified from FA by image analysis with Photoshop. Some eyes were collected for histologic analysis.

RESULTS. IVT AAA produced neuronal degeneration over a large fraction of the retina. RNV formed in the damaged area and by 10 weeks exhibited stable morphology and leakage, which persisted for at least 65 weeks. Control IVT injections did not affect RNV leakage, but IVT aflibercept completely blocked RNV leakage. The inhibition was reversible (i.e., the leakage returned as the drug cleared), and the duration of antileak effects with 500 μg aflibercept was approximately 8 weeks. Partial regression of the pathologic vasculature also occurred with aflibercept, with reestablishment as the drug cleared.

CONCLUSIONS. This model mimics a chronic human disease in its stability and persistence, and the antileak action of aflibercept is fully reversible with a dose-dependent duration. Therefore, this large eye model is uniquely suitable for investigations into the efficacy and duration of action of novel formulations and pharmacotherapies for retinal vascular diseases, and for studying the underlying pathobiology of retinal angiogenesis.

Keywords: retinal vasculature, neovascularization, leakage

Pathologic vessel growth and vascular leakage have a role in several common blinding eye disorders. In neovascular age-related macular degeneration (nAMD), vessels from the choroid grow inappropriately through Bruch’s membrane into the subretinal pigment epithelium (RPE) and subretinal space. These vessels are leaky and fragile, leading to fluid accumulation and hemorrhage, thereby damaging vision. In diabetic retinopathy (DR), hyperglycemic damage to the retinal microvasculature leads to ischemia and inflammation that drives pathologic fluid accumulation (diabetic macular edema, DME) and/or angiogenic proliferation in the inner retina (proliferative diabetic retinopathy, PDR). Partial or complete vision loss may result from the ensuing retinal swelling, hemorrhage, and/or tractional detachment. The medical treatment of these, and several other retinal vascular disorders, has been revolutionized in the past decade due to the use of biological therapeutics that bind to and neutralize VEGF, a key driver of physiologic and pathologic endothelial cell activation and proliferation. The first of these, pegaptanib, is an RNA-based aptamer that was approved for use in the United States for nAMD in 2004. Superior clinical results were achieved with ranibizumab, an antibody Fab fragment, and aflibercept, a receptor/antibody chimeric fusion protein, approved in 2006 and 2011, respectively. Bevacizumab is a full-length antibody against VEGF, approved for use in several cancers, that is widely used off-label to treat ophthalmic vascular disorders. Anti-VEGF therapeutics, administered via intravitreal (IVT) injection, currently are the standard of care in the European Union and the United States for treatment of retinal vascular occlusive disease (RVO), nAMD, and DME.

Despite these advances, unmet medical needs for improved therapies remain. While the majority of patients have clinical success, there are patients whose response is limited with anti-VEGF monotherapy. For example, in the VIEW 1 & 2 studies, approximately 95% of patients treated with either IVT aflibercept or ranibizumab maintained their vision (defined as losing fewer than 15 letters on the standardized Early Treatment Diabetic Retinopathy Study (ETDRS) chart from baseline to Week 52. However, only approximately one-third of patients achieved an improvement of 15 or more ETDRS letters in best corrected visual acuity (BCVA), suggesting that a significant proportion of patients had the potential to gain additional vision. Similarly, approximately one-third of patients with DME demonstrated a significant gain of 15 ETDRS letters at Week 52 in the VISTA and VIVID studies when treated with IVT aflibercept.
Each clinical trial used monthly or bimonthly IVT injections, which represent a significant treatment burden on patients, caregivers, and physicians. While several alternate dosing regimens have been proposed and used with varying success,14-18 measures of real world clinical improvement indicate much poorer outcomes than in the trials, which is likely due to poor compliance with recommended treatment regimens, that is, undertreatment.19-20 Hence, there is a pressing need for more effective and durable therapies in the real world of clinical practice.

One barrier to the discovery and development of better therapies for retinal vascular diseases is the lack of large eye animal models that mimic the chronic nature of these human diseases. The laser-induced choroidal neovascularization (CNV) model in mouse or nonhuman primate is self-limiting because a large fraction of the injury-induced neovascular leakage permanently heals in a matter of weeks.21-25 Anti-VEGF therapy, even in quite low doses, accelerates this healing (unpublished data). Therefore, new formulations and therapies for this leakage by aflibercept. This model provides the unique ability to benchmark experimentally novel agents, combinations of agents, and formulations in terms of efficacy and duration of action relative to standard-of-care anti-VEGF agents.

AAA Preparation

A 120 mg amount of AAA (Lot: BVBG0603V; Sigma-Aldrich Corp., St. Louis, MO, USA) was dissolved in 1 mL hydrochloric acid [1 N] (VWR, Radnor, PA, USA). This AAA stock was diluted to an 80 mM solution using 0.9% sterile normal saline solution and was brought to a pH of 7.4. The final solution was passed through a disposable Millex-GP syringe filter unit with a pore size of 0.22 μm (Batch, MKBL8989; Sigma-Aldrich Corp.) to remove any potential particulates. Solutions were made immediately before use and all solutions remained at room temperature until time of injection.

Induction of Retinal Neovascular Leakage

Initial baseline in-life ophthalmic evaluations were performed before induction of RNV. Rabbits were anesthetized with ketamine (35 mg/kg, intramuscular) and Xylazine (5 mg/kg, intramuscular). Heart rate, respiratory rate, mucus membrane color, body temperature, and pulse oximetry were monitored every 15 minutes for the entire duration of anesthesia in each animal. Corneas were anesthetized further using a 0.5% ophthalmic solution of proparacaine hydrochloride (Bausch+Lomb, Garden City, NY, USA). Pupils were dilated using a 1% ophthalmic solution of tropicamide (Bausch+Lomb). An additional drop of a GenTeal (Alcon, Fort Worth, TX, USA) lubricating eye gel was applied to the eye to help with corneal hydration. A juvenile ophthalmic speculum then was used to open the eyelids for intraocular imaging (VWR).

Ophthalmic evaluations consisted of a photograph of the eye using a Canon PowerShot digital camera (Canon, Tokyo, Japan) for assessment of gross inflammation and an IOP measurement using a Tono-Pen (Reichert, Depew, NY, USA), before pupil dilation. Approximately 5 minutes after pupil dilation, fundus examination using a WelchAllyn PanOptic Ophthalmoscope (Skaneateles Falls, NY, USA), Red-free Imaging using a Spectralis Heidelberg retinal angiography platform HRA+OCT system (Heidelberg Engineering, Carlsbad, CA, USA), early (0–3 minutes) and late (10–13 minutes) phase fluorescein angiography (FA) using the Spectralis imaging system, and multiple 61-scan P-Pole optical coherence tomography (OCT) imaging using the Spectralis system were performed for each eye.

Following initial baseline in-life ophthalmic evaluations, male NZW rabbits received an 80 μL IVT injection of an 80 mM AAA solution (described earlier) with an injection site at 10 o’clock for the right eye (OD) and 2 o’clock for the left eye (OS). After 10 minutes, a second IOP measurement was obtained to assess acute pressure changes due to injection volume. An additional ophthalmoscope observation was used to identify any potential damage during injection. A 0.5% Erythromycin Ophthalmic Ointment (Bausch+Lomb) was applied to the eye immediately after observation.

Animals

Male New Zealand White (NZW) rabbits with a mean age of 8 to 10 weeks and weight range of 2 to 2.5 kg were purchased from Millbrook Labs (Amherst, MA, USA) or Charles River Laboratories (Wilmington, MA, USA). Animals were housed in pathogen-free conditions at the animal facilities of Regeneron Pharmaceuticals, Inc. (Tarrytown, NY, USA) or at the Department of Comparative Medicine at New York Medical College (Valhalla, NY, USA). All animals were maintained with a room temperature between 18°C and 26°C (64°F–79°F), a relative humidity between 50% and 70%, and a 12-hour light-dark cycle. Animals were fed a Purina 5326 lab diet and acidified water, ad libitum. All animals were treated humanely; all animal-related procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Regeneron Pharmaceuticals, Inc. and New York Medical College, and all experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Labeling of Proliferating Vascular Cells With Bromodeoxyuridine (BrdU)

NZW rabbits received two IVT injections of BrdU (Sigma-Aldrich Corp.) at 10 mcg/50 μl, on days 28 and 32, after DL-AAA. At week 10, rabbits were euthanized and perfused with fluorescein ConA (Vector Laboratories, Burlingame, CA, USA)
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In the study, rabbits were divided into treatment or control groups. Rabbits in the treatment groups received IVT aflibercept at 250 μg/50 μL/eye with 10% sodium fluorescein (12 mg/kg; AK-FLUOR10%; Alcon, Fort Worth, TX, USA) for 3 hours at 37°C and mounted with ProLong antifade (Molecular Probes, Eugene, OR, USA).

In-life Imaging and Quantification of Vascular Leakage

Red-free images were taken using a fundus camera in a Spectralis Heidelberg retinal angiography platform HRA+OCT system (Heidelberg Engineering). The red-free images were taken of the temporal, central, and nasal nerve fiber layers of each eye. The animal then was injected in the marginal ear vein with 10% sodium fluorescein (12 mg/kg; AK-FLUOR10%; Alcon, Fort Worth, TX, USA) for FA imaging using the same Heidelberg Spectralis machine. Early (0–3 minutes) and late (10–13 minutes) phase time point images were acquired for analysis.

For OCT imaging, 61 horizontal sections were obtained temporal and inferior to the optic nerve head, the location of retinal degeneration. An additional series of 61 horizontal sections were obtained nasal and inferior to the optic nerve head, typically a location of intact, healthy retina. A final series of 61 vertical sections were obtained near the temporal periphery of each eye using the OCT function on the Spectralis HRA+OCT system (Heidelberg Engineering).

Red-free images and early-phase FA images were exported from the Heidelberg software and imported to Adobe Photoshop CC. Multiple images per eye were overlaid and merged into a mosaic of the fundus. FA images, leakage area was quantified by tracing over the fluorescein cloud in the vitreous using a paintbrush tool and calculating the number of pixels covered. Leakage area was standardized weekly using the area of the optic nerve head. Data were recorded as the percent leakage area when compared to baseline leakage area before any treatment with aflibercept.

Statistical Analysis

At each time point, percent leakage area was compared among treatments using 1-factor ANOVAs with a Tukey’s multiple comparison test. All analyses were performed using GraphPad Prism (Version 5.0a; GraphPad Software, Inc., San Diego, CA, USA). Data are shown as mean values ± SEM, unless stated otherwise. A P value of less than 0.05 was considered statistically significant.

Measurements of Vitreal VEGF

Vitreous was isolated and centrifuged for 10 minutes at 10,000g from normal and DI-AAA treated eyes with already established disease. The upper phase was collected, aliquoted, and stored at −80°C until VEGF levels were assessed. VEGF levels were measured using a Milliplex Assay from Millipore (Billerica, MA, USA) following manufacturer’s instructions.

Histology and Immunohistochemistry

Eyes were enucleated and placed in either 10% formalin or Davidson’s fixative for 48 hours. Following fixation, right eyes were dissected and placed in 70% ethanol until processed for paraffin embedding. Serial sections from each eye then were stained with hematoxylin and eosin.

Left eyes processed for immunostaining were embedded in OCT Tissue-Tek (Sakura, Torrance, CA, USA), sectioned, and stored at −80°C. Before washing the OCT with PBS (Gibco Thermo Fisher Scientific, Waltham, MA, USA), the eyes were placed in a 50% to 60°C oven for 15 minutes. Following removal of OCT, the tissue was permeabilized with 0.1% Triton X-100 (Thermo Fisher Scientific) for 15 minutes and blocked with PBS+1% BSA (Sigma-Aldrich Corp.) +0.1% TritonX-5% normal goat serum (Vector) for 1 hour. Mouse anti-B-Tubulin Alexa488 (Biolegened, San Diego, CA, USA) was added at 1:200 in blocking buffer and sections were incubated at 4°C overnight. The following day, sections were washed with PBS and mounted with ProLong Gold Antifade (Molecular Probes). Images were acquired in a Nikon 80i Eclipse Microscope (Nikon, Melville, NY, USA).

RESULTS

IVT AAA Caused Retinal Degeneration and Vascular Pathology

Following IVT injection of AAA, retinal disorganization and degeneration occurred over the next several weeks (Fig. 1). This was characterized initially by a damaged retinal surface with inflammatory cell infiltration in the vitreous, disorganized retinal ganglion cell layer, loss of inner limiting membrane, fewer nuclei in the inner nuclear layer, and areas of irregular photoreceptors. The retina gradually became thinner, as evidenced by OCT imaging and confirmed by histology, and by 8 weeks after injection virtually no neurons remained; only lingering glial and inflammatory cells were present. (Figs. 1G,
**FIGURE 1.** IVT AAA injection leads to retinal degeneration. Histologic (A, C, E, G) and OCT (B, D, F, H) images at different time-points (pre-injection, 1, 4, 8 weeks) after IVT injection of AAA. Invading leukocytes (arrows in [C]) and retinal degeneration are apparent, and the OCT image shows intraretinal separation (arrow in [D]) 1 week after injection. Retina thinning continues and by week 8 there are no neurons remaining in the damaged area.
FIGURE 2. Loss of ganglion cell axons and medullary ray structure after IVT AAA. Gradual and complete retinal degeneration in the AAA-affected area is apparent by the ganglion cell axonal loss evident by 2 weeks after AAA injection with complete loss by 8 weeks.
FIGURE 3. Development of vascular pathology in the rabbit retina during the weeks after IVT AAA. Red-free fundus photography (left) and FAs (right) illustrate the pattern of vascular leakage and abnormalities after IVT AAA. During the first 2 weeks, there is extensive leakage from dilated and tortuous vessels with the beginnings of angiogenic growth centrifugally toward the retinal periphery. After 4 and 6 weeks, the leakage is present but diminished and the neovessels continue to extend. After 8 weeks, the vessels remain tortuous and dilated, with a florid, extended, but stable structure. Vascular leakage is robust but restricted to the tips of the telangiectatic neovessels (arrows). Areas specified by the boxes at week 8 are shown enlarged in the bottom two figures.
The loss of ganglion cells in the degenerated area is evidenced by the gradual disappearance of the medullary bundles, which in a normal eye contains the myelinated axons of these cells (Fig. 2).

Within a few days after IVT injection, AAA produced local retinal neuronal and vascular damage as shown histopathologically and in the OCT image (Figs. 1C, 1D). Fluorescein angiography revealed damage-related leakage at weeks 1 and 2, which decreased spontaneously within 2 to 4 weeks without any treatment (Fig. 3, weeks 1, 2, and 4). This pattern of retinal neural degeneration and vascular pathology occurred over a large but incomplete fraction of the retina, usually the injected, temporal side.

At later time points, a different pattern of pathology emerged. RNV formed in the damaged area over 6 to 8 weeks (Fig. 3, weeks 6 and 8), which appeared as multiple irregular, dilated, and tortuous blood vessels derived from superficial retinal feeder vessels normally over or in-between the bundles of medullary nerve fibers (Fig. 3). A view of the entire retinal vasculature is presented in Figure 4. For this specimen, the

**FIGURE 4.** Rabbit retinal vasculature in a flat mount preparation. (A) In-life labeling of perfused vasculature with fluorescein Concanavalin A. (B) External labeling of the retinal vasculature with Rhodamine *G. simplicifolia* lectin. (C) Merge of (A) and (B). These images illustrate the morphologic changes that occur in the retinal vasculature after DL-AAA. On the temporal, injected, side of the retina (right), the blood vessels are dilated, tortuous, and end in tufts.
rabbit was perfused with 1% paraformaldehyde containing fluorescent concanavalin A (green) to bind to vascular endothelial cells and thereby label the luminal surface of vessels before fixation. The retina then was whole-mounted, and incubated with fluorescent *Griffonia simplicifolia* lectin I (red) to label the external surface of the vessels. The anatomic differences between the vessels on the normal side and the tortuous, dilated, extensively branched ones on the injected side are readily apparent. Since all vessels are colabeled with luminal and abluminal markers, all vessels, normal and pathologic, are patent and perfused. The increased length and larger area covered by the vessels is evidence angiogenesis has occurred and that these are true neovessels. To further prove that these vessels were born recently by endothelial cell proliferation, we injected BrdU IVT twice, at days 28 and 32 after AAA administration. We would expect that any cells dividing at that time would take up the BrdU and permanently incorporate it into their nuclear DNA in place of thymidine. That endothelial cell proliferation was occurring is apparent by staining with an antibody to BrdU (Fig. 5) after euthanization at week 8 after AAA. BrdU-positive nuclei are found in endothelial cells along a stretch of vessel far distal to where normal rabbit retinal vessels are found. The appearance of the fundus as monitored by four common imaging techniques, color photography, red-free imaging, infrared imaging, and FA, are shown in Figure 6.

A highly characteristic pattern of vascular leakage emerged between weeks 6 and 8 after AAA. The leakage occurred exclusively at the extended tips of new vessels, evidenced by red-free or infrared imaging and fluorescein angiography (Fig. 5).
Vascular morphology and patency were normal on the undamaged side (Figs. 4, 6). By 7 to 8 weeks after AAA, the RNV had a stable morphology and extent of leakage. Notably, the vascular pathology and leakage were qualitatively and quantitatively stable for at least 65 weeks (Fig. 7).

**Effects of Aflibercept on Retinal Vascular Leakage**

VEGF levels in the vitreous of normal and AAA rabbit eyes were measured using a Milliplex kit. Levels were below the level of quantitation (~1 ng/mL) in control vitreous and 12.9 ± 2 ng/mL in AAA vitreous (20 weeks after AAA).

**FIGURE 6.** Retinal vascular pathology in the AAA model is evident using common fundus imaging modalities. (A) The loss of medullary rays is very apparent with color fundus photography, as the reflectance from the myelin sheaths is gone and choroidal vessels are fully visible on the damaged side. (B) Red-free photography provides the highest contrast and, therefore, most articulated image of the vasculature on normal and pathologic sides. (C) Infrared also provides excellent visualization of the pathologic vasculature and has been used for many experiments. (D) Fluorescein angiography permits characterization and quantitation of vascular leakage. The characteristic pattern of fluorescein leakage from the tips of the new vessels is apparent (arrows in [A–D]).

**FIGURE 7.** Vascular leakage is highly stable for more than 1 year. FA images from the same retina at intervals after IVT injection of AAA starting at 7 through 65 weeks. Qualitative and quantitative features of the leakage are very well-maintained.
Aflibercept completely and reversibly inhibits pathologic vascular leakage. (A) Red-free (left column, [a–d]) and FA (middle column, [e–h], and right column, [i–l]) images of AAA-treated retinas at baseline (i.e., 8 weeks after AAA treatment) and at 1, 8, and 20 weeks after an IVT injection of aflibercept (500 μg). The region of the box in the middle image is shown at higher power on the left and right-side images. Leakage and tortuous vessels are visible at baseline (a, e, i). One week following aflibercept, the leakage is completely inhibited, tortuosity has decreased, and the finer vessels are difficult to visualize if present (b, f, j). Eight weeks after aflibercept treatment, leakage begins to return (c, g, k). By 20 weeks after aflibercept treatment (d, h, l), leakage and vessel morphology have returned to baseline levels. (B) Quantitation of the leak area is provided in the plot. Data are means ± 1 SEM. (C) Injection of 250 μg aflibercept led to 100% inhibition of baseline leak measured at week 1 after injection, which fully recovered by week 16. A second injection (at a lower dose of 50 μg) again led to 100% inhibition measured the following week.
IVT aflibercept completely blocked RNV leakage (compare Figs. 8A-c, 8A-i with Figs. 8A-f, 8A-j), but treatment with buffer or human Fc had no effect (not shown). The pathologic vessels became narrower and less tortuous after aflibercept treatment, with apparent disappearance or lessened visibility via imaging, of the leaky tips and finer processes (compare Fig. 8A-a with Figs. 8A-b, 8A-c). The inhibition was reversible (i.e., the leakage returned as the drug cleared; Figs. 8A-h, 8A-i), which was confirmed by an in-house pharmacokinetic study (data not shown).

The duration of the antileak effect was dose-dependent, with the highest dose of 500 μg of aflibercept providing 7 to 8 weeks of complete inhibition, while a dose of 125 μg provided 2 to 3 weeks of complete inhibition (Fig. 8B). After 20 weeks, when the leakage had returned to predrug levels, the morphologic pattern of the vessels appeared very similar to that seen before aflibercept injection (compare Figs. 8A-a and 8A-d). A second injection of aflibercept (50 μg, in AAA eyes injected with 250 μg aflibercept initially) again resulted in complete inhibition of leakage (Fig. 8C). Detailed duration of action or dose-response studies have not yet been performed for repeat injections.

DISCUSSION

We described a new rabbit model of persistent RNV and vascular leakage, the AAA model. We do not claim that this model shares any strict anatomic or genetic features with exudative AMD (e.g., there is no chorioidal neovascularization) or DME (e.g., there is no edema; indeed, there is only a residual neural retina in the area of the RNV and no contribution from hyperglycemia), which are the two largest disease indications and mechanisms and therapies. These features include the large size of the eye, remarkably stable nature of the pathology, additional due to the stability of vascular pathology in the AAA model, it may be useful for analyzing the mechanisms of neovessel maintenance, particularly when morphology is maintained for over a year.

In summary, the rabbit AAA model of sustained retinal vascular pathology has unique features that render it highly useful for the investigation of retinal angiogenic disease mechanisms and therapies. These features include the large size of the eye, remarkably stable nature of the pathology, susceptibility to anti-VEGF therapies, and reversible nature of the effects of inhibitors.

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