A novel model of persistent retinal neovascularization for the development of sustained anti-VEGF therapies

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Abstract
Anti-vascular endothelial growth factor (VEGF) therapies lead to a major breakthrough in
treatment of neovascular retinal diseases such as age-related macular degeneration or dia-
betic retinopathy. Current management of these conditions require regular and frequent
intravitreal injections to prevent disease recurrence once the effect of the injected drug
wears off. This has led to a pressing clinical need of developing sustained release formul-
ations or therapies with longer duration. A major drawback in developing such therapies is
that the currently available animal models show spontaneous regression of vascular leakage.
They therefore not only fail to recapitulate retinal vascular disease in humans, but also pre-
vent to discern if regression is due to prolonged therapeutic effect or simply reflects sponta-
naneous healing.

Here, we described the development of a novel rabbit model of persistent retinal neovascu-
larization (PRNV). Retinal Müller glial are essential for maintaining the integrity of the blood-
retinal barrier. Intravitreal injection of DL-alpha-aminoadipic acid (DL-AAA), a selective reti-
nal glial (Müller) cell toxin, results in persistent vascular leakage for up to 48 weeks. We
demonstrated that VEGF concentrations were significantly increased in vitreous suggesting
VEGF plays a significant role in mediating the leakage observed. Intravitreal administration
of anti-VEGF drugs (e.g. bevacizumab, ranibizumab and aflibercept) suppresses vascular
leakage for 8 to 10 weeks, before recurrence of leakage to pre-treatment levels. All three
anti-VEGF drugs are very effective in re-ducing angiographic leakage in PRNV model, and
aflibercept demonstrated a longer duration of action compared with the others, reminiscent
of what is observed with these drugs in human in the clinical setting. Therefore, this model
provides a unique tool to evaluate novel anti-VEGF formulations and therapies with respect
to their duration of action in comparison to the currently used drugs.

1. Introduction
Pathological retinal neovascularization is a common complication of many ocular diseases,
such as exudative age-related macular degeneration, pathologic myopia, proliferative dia-
betic retinopathy (De Schaepdrijver et al., 1989) and retinopathy of prematurity (ROP)
which often result in blindness (Cheung et al., 2010; Koh et al., 2012; Tan et al., 2017; Wong
et al., 2016; Wong and Scott, 2010). The mammalian retina receives its nutrition from two
discrete circulatory systems. The arteries of the uveal circulation provide the greatest blood
flow and supply to the middle and outer layers of the retina. The inner retina, on the other
hand, derives its circulation from the central retinal artery. There are mainly two types of
retinal neovascularization that differ depending on the location from where the new vessels
sprout.

Retinal neovascularization that occurs in PDR and ROP develops within the retinal vascular
network; new vessels sprout from the retinal circulation and invade into the vitreous and
neural (inner) retinal layers and the vitreous (Wong et al., 2016; Wong and Scott, 2010).
Choroidal neovascularization (CNV), a key feature of neovascular age-related macular de-
generation (nvAMD) occurs when new blood vessels begin to grow from choroidal blood
vessels, breaking through the Bruch’s membrane under the RPE, into the subretinal space.
Because these choroidal vessels that invade into the retina are immature, lacking pericyte
integration and intercellular tight junctions, they are characterized by increased vascular
permeability that results in sub-retinal edema (Tornquist et al., 1990).
Both retinal and choroidal neovascularization share common clinical features of neovascularization, and through retinal vascular leakage, lead to retinal edema, hemorrhage and fibrosis, ultimately causing visual impairment and blindness (Al-Shabrawey et al., 2013; Grossniklaus et al., 2010). The discovery that vascular endothelial growth factor (VEGF) is a key pathogenic factor in RNV and CNV, has led to the intravitreal administration of anti-VEGF agents providing an important breakthrough in the treatment of patients with neovascular AMD, diabetic macular edema, retinal vein occlusion and proliferative diabetic retinopathy, leading to an improvement on patient outcomes (Cheung et al., 2010; Koh et al., 2012; Tan et al., 2017; Wells et al., 2016; Wong et al., 2016; Wong and Scott, 2010). However, a key limitation with current anti-VEGF therapies in clinical use (bevacizumab, ranibizumab and aflibercept) is its short duration effect.

Consequently, optimization of patient outcome with these agents requires intensive disease monitoring with frequent intravitreal injections. Thus, there is a large unmet medical need for the development of longer lasting anti-VEGF therapies or other agents that can cause an involution of the pathologic retinal neovascularization. An animal model of persistent retinal neovascularization with chronic increased vascular leakage could facilitate these efforts. The most common experimental model for nvAMD in retina is laser-induced CNV. In both rodents and non-human primates, vascular leakage is typically observed for 2 to 3 weeks after laser photocoagulation, but lead to regression and cease to leak with no longer follow-up (Miller et al., 1990; Shen et al., 2004). Thus, this model provides a relatively short term window for the evaluation of anti-neovascular or anti-vascular permeability agents or other agents that have the potential to cause the involution of pathological vessels. Following intravitreal injections of anti-VEGF, vascular leakage is almost completely eliminated in these models (Husain et al., 2005; Nork et al., 2011), but in human, recurrence of leakage and edema often occur. The exogenous intravitreal administration of recombinant VEGF in Dutch Belted rabbits is another frequently used model to demonstrate increased vascular permeability. While the effects of VEGF on vascular permeability can persist 4 to 5 days and need to repeat challenge weekly, the effects of VEGF on vessel permeability are markedly decreased with subsequent rechallenged (Edelman et al., 2005).

Retinal Müller cells play an essential role in regulating neuronal activity and maintaining the integrity of the blood-retinal barrier. DL-alpha-amino adipic acid (DL-AAA) is a well-known selective retinal glial (Müller) cell toxin (Olney, 1982). Subretinal injection of DL-AAA has been shown to disrupt retinal Müller cells, induce vascular telangiectasis and increase vascular permeability with increased expression of VEGF and the reduced expression of the tight junction protein in rodents (Shen et al., 2010). However, there are no prior studies evaluating these properties in response to anti-VEGF treatments.

Therefore, we developed a retinal neovascular model with human disease feature, including a persistent retinal neovascularization (PRNV) by a single intravitreal injection of DL-AAA into Dutch-Belted pigmented rabbits. We performed detailed characterization of this model, including imaging and histology. Subsequently, we evaluated the role of VEGF in this model by assessing the response to anti-VEGF therapy using current anti-VEGF therapies in clinical use (bevacizumab, ranibizumab and aflibercept). The clinical feature of PRNV with chronic
retinal exudation as described in this study appears to be a better model for assessing the therapeutic potential of long-acting anti-neovascular or anti-vascular permeability agents.

2. Methods

2.1. Animals

Adult, Dutch Belted pigmented rabbits weighing 1.5-2.0 kg were obtained from Covance (Covance Research Products Inc., Denver, PA). All animals were acclimatized for at least a week before the start of the experiment. All experiments with animals were designed and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the Singhealth Experimental Medicine Center.

2.2. Intravitreal Injection

PRNV was induced with a single intravitreal injection in one eye with 50μl of 0.025M DL-AAA whereas 50μl of saline was injected into the contralateral eye as control. Prior to injection, both eyes were topically anesthetized with 1% lignocaine drops to minimize discomfort and were treated with a topical antibiotic ointment (Tobrex® Tobramycin 0.3%; Alcon) thereafter.

The anti VEGF agents were masked as A, Aflibercept 1.2mg/eye (Eylea®, Regeneron, Tarrytown, New York, USA and Bayer HealthCare); B, Ranibizumab 0.3mg/eye (Lucentis®, Genetech Inc., South San Francisco, CA, USA and Novartis) and C, Bevacizumab 0.75mg/eye (Avastin® Roche Diagnostics GmbH, Mannheim, Germany) in 30µl for intravitreal injection.

2.3. Imaging

Non-invasive ocular imaging including fundus photography (TRC-50DX, TopCon Corp., Tokyo, Japan), fluorescein angiography (FA) and optical coherence tomography (OCT) were performed using Heidelberg Spectralis® HRA + OCT (Heidelberg Engineering, Heidelberg, Germany) at designated time points over a period of six months including baseline. All images were evaluated using the Heidelberg Eye Explorer software (HEYEX, version 1.7.1.0). All imaging procedures were done in conscious animals and pupils were dilated with 1 drop of 2.5% Phenylephrine HCl Ophthalmic Solution and 1 drop of 1% Tropicamide (Alcon Laboratories Inc, Fort Worth, TX) before every imaging.

PRNV angiograms were graded to assess the size of leak area and intensity after 8 to 10 weeks from the onset of PRNV induction. The leak area and intensity were defined by fluorescein angiography (NaF 100mg/ml, Novartis). Stable angiographic leak area of at least 3 disc diameters and leak severity of ≥ 3 on the 0–4 scale were screened out for pharmacology study. The assessment was based on clinical masked scoring (0 = no leak, 1 = minimal leak, 2 = mild leak, 3 = moderate leak, 4 = severe leak) while mean leak areas (MLA) was measured by image analysis based on brightness of the optic disc blood vessel using open-source software (ImageJ).

2.4. Measurement of VEGF-A levels with ELISA

Vitreous and aqueous samples were obtained from 8 Dutch-Belted Rabbits induced with PRNV (OD), and contralateral naive eyes (OS). Samples from the PRNV eyes (n=4) and naive eyes (n=4) were collected at 8 weeks after DL-AAA intravitreal injection. The concentration of VEGF-A in the vitreous and aqueous were quantified via enzyme linked immunosorbent
assay (ELISA) using Human Premixed Multi-Analyte Kit (R&D Systems, Inc. Minneapolis, MN, USA) according to the manufacturer's protocol. The limits of Quantification of VEGF-A were min = 0.9465 pg/mL and max = 2051 pg/mL.

This experiment was repeated twice in duplicates. 50ul per well of either vitreous or aqueous were incubated with human magnetic microparticle beads that are pre-coated with the VEGF-A antibody. The plates were washed using a magnetic plate separator (Bio-Rad, USA) with subsequent incubation of human premixed biotin antibodies cocktail. Reactions were then incubated with Streptavidin-PE before being read using Bio-Plex 200 system (BioRad, CA, USA). Positive controls were set up for both recombinant human (R&D Systems, Inc. Minneapolis, MN, USA) and rabbit VEGF-A (Kingfisher Biotech, Inc. USA) as a means to show that VEGF-A levels detected in rabbit samples is comparable to human samples.

2.5. Histological and Immunohistochemical Staining
Two rabbits were sacrificed at week 24 post DL-AAA intravitreal injection for histologic examination. Both eyes were immediately enucleated and fixed in Carnoy's Solution (Electron Microscopy Services, USA-Hartfield, PA) overnight. The tissues were processed, embedded in paraffin then sectioned at 5 μm thickness using the Leica Microtome RM2255 (Leica Biosystems Nussloch GmbH, Germany). Serial sections were stained with hematoxylin-eosin (Modified Mayer’s ab220365, Abcam Singapore) using an automatic stainer (Leica Autostainer XLR, Leica Biosystems Nussloch GmbH, Germany). The expression of glial fibrillary acidic protein (GFAP) was stained with anti-GFAP (Mouse monoclonal GA5 ab212398, Abcam Singapore) (1:200 dilution) for 90 minutes at room temperature and then visualized by diaminobenzidine (DAB) reaction. Appropriate positive and negative procedural controls were used throughout.

2.6. Clinical assessment for pharmacology study
To determine the effect of the treatments, the rabbits were evaluated with non-invasive imaging techniques. Retinal images and fluorescein angiograms were obtained with the Heidelberg Spectralis® HRA + OCT. Rabbit pupils were dilated with 1 drop of 2.5% Phenylinephrine HCl Ophthalmic Solution and 1 drop of 1% Tropicamide (Alcon Laboratories Inc, Fort Worth, TX). Sequential fundus photographs were taken immediately after 0.5 ml 10% Sodium fluorescein was injected via the marginal ear vein. Sensitivity was set from 30-41 and a minimum of 3 scans was obtained from each eye from different locations. Late-phase angiograms of the diseased eye were obtained within 1 to 3 minutes after IV injection including the central, nasal and temporal images with the optic nerve head in the mid-horizontal plane. Nasal and temporal images bisected the optic disc at the edge of the frame. Late-phase angiograms of the contralateral eye were also obtained immediately after imaging the diseased eye.

Retinal structural changes were assessed using OCT horizontal 25-line raster A-scans. Approximately 5000µM was measured from the ventral optic nerve head while choosing the section which best corresponds to the distance. The line scans were taken at all areas below the optic disc and medullary wings. A standard slit lamp (Righton RS-1000. Right MFG., Co, Ltd, Japan) was also used for imaging to identify inflammatory and immune response for the anterior segment.
Follow-up with colour fundus photography and neovascular angiographic leak area with FA were obtained at baseline before dosing and subsequent 1, 2, 4, 6, 8 and 10 weeks. Angiograms were exported in the jpeg format and analyzed. The leak area was quantified from the angiograms by using the analysis program of ImageJ. The MLA was measured by quantifying the number of encompassed pixels. The methodology that was utilized to extract the leakage area outputs repeatable and robust quantitative figures. This algorithm works in 3 steps. First, the image is converted to 8-bit grayscale and multiple points on the primary (larger) vessels are selected. The whole image is then thresholded based on these points. Then a vessel identifier algorithm extracts the vasculature bed from the image. This course of action provides the extracted leakage area from the vasculature network (Fig. 8). The number of pixels within this area is measured and monitored over time.

2.7. Statistical analysis
Statistical analysis was performed by using the Prism 5 GraphPad Prism Software (La Jolla, CA, USA) and data analyzed with unpaired t-test for comparison of two samples and Two-way ANOVA for comparison of three samples or more. Results are expressed as mean ± SD. p ≤ 0.05 as statistically significant.

3. Results
3.1. PRNV development was induced by a single intravitreal injection of 0.025M DL-AAA. Persistent leakage of PRNV was demonstrated up to 48 weeks.

One week after injection, the DL-AAA-injected eyes demonstrated prominent retinal vasodilatation, vessel tortuosity and retinal edema. Neovascularization was observed as early as 2 weeks after the DL-AAA injection as evidenced by engorged retinal vessels growing into the visual streak area from the vasculature of the optical disc (Fig.1A). The FA features of the neovascularization were characterized by well-demarcated boundaries discernible at an early phase of angiography and progressive leakage in later phase of angiography (Fig. 1B). DL-AAA injection produced clinical PRNV as revealed by FA with retinal disorganization in the visual streak. The hyperfluorescent leaking lesion increased rapidly in the first 4 weeks, relatively stabilized within 4 to 8 weeks, and was persistent throughout the entire observation of 12 months without regression. The incidence of clinical PRNV, indicated by fluorescein leakage was 85% (55/65 eyes) at 8 weeks following DL-AAA administration. PRNV typically developed within the visual streak, sometimes extending in a non-symmetrical pattern from the medullary rays into nasal or temporal aspects. In total, there were 51% (28/55) PRNV in the central area and 49% (27/55) inclined to one side in non-symmetrical pattern.

Morphological retinal changes were evaluated by non-invasive retinal imaging using SD-OCT. The OCT images were taken through the visual streak where PRNV was seen in most DL-AAA-treated eyes. DL-AAA injection caused extensive intraretinal fluid within 4 hours and retinal thickening plus accumulation of fluid within 24 hours. Internal limiting membrane (ILM) thickening was observed between 24 hours and Week 1. By the first week, bullous schisis and loss of outer retina was noted in some sections. There were marked disruption of entire retina, retinal thinning and retinal laminat ion loss instead of scar and fibrovascular deposition on superficial retina where the PRNV can be easily seen from OCT images from 4 weeks onwards (Fig. 2).
3.2. Intravitreal DL-AAA led to retinal damage, PRNV development in central retina and GFAP activation in Müller cell in peripheral retina

Histological changes were examined by light microscopy. Photomicrographs of retinal sections from a representative PRNV rabbit is shown in Fig 3. The right eye which was treated with 0.025M DL-AAA at 24 weeks and left eye with saline as controls were stained with hematoxylin-eosin (H&E) and glial fibrillary acidic protein (GFAP). Intravitreal DL-AAA triggered retinal damage in different regions of the eye from central to peripheral retina. In the central retina (Fig.3A, C and D), DL-AAA induced a localized disruption in the visual streak, including thinning of the external and internal plexiform layers, and disruption of the outer and the inner nuclear layers. The ganglion cell layer was essentially absent. New blood vessels (arrows in Fig.3A and D) have developed in the surface of the disruptive retina and have grown outward into the vitreous cavity. In comparison, the peripheral region and the retinal structure mainly remain intact in the DL-AAA eyes, and no abnormal histopathological changes were observed in the saline-injected eyes (Fig.3G).

Under normal conditions, Müller cells express little or no GFAP (Fig.3H) but become strongly GFAP-immunoreactive in dystrophic retinas under pathogenic conditions (Bringmann et al., 2006). Similar changes in locations to H&E staining, GFAP staining (brown color, Fig.3E and F) demonstrated that GFAP immunoreactivity on Müller cells was mildly increased in the peripheral region while strong GFAP immune-labeling was observed in the visual streak of DL-AAA-treated eyes, indicating Müller cell damage after the DL-AAA injection.

3.3. Intravitreal aflibercept, ranibizumab and bevacizumab are very effective in reducing angiographic leakage in PRNV model, suggesting VEGF plays an important role in this model

The effect of three commercially available anti-VEGF agents on angiographic leakage was evaluated by late-phase FA at 1, 2, 4, 6, 8 and 10 weeks after each intravitreal injection. All PRNV eyes (n=18) of the three treatment groups (n=6 /each group) showed an absence of angiographic leakage from the PRNV sites at 1 week post treatment. The duration of the effect in inhibition of the leakage in FA varied with the three Anti-VEGF agents. The recurrence of leakage mainly started at 6 weeks with ranibizumab (Drug B) group, 8 weeks in bevacizumab (Drug C) group and 10 weeks using aflibercept (Drug A)(Fig. 5a).

Figure 4b show the graphical representation by percent change in mean leakage area (MLA) of each group following anti-VEGF treatment over time. Mean pre-anti-VEGF treatment total leakage was normalized as % of baseline in Drug-A, B and C groups, respectively. Between week 2 and week 8, MLA was significantly reduced from baseline in all 3 groups. Difference in the duration of effects on MLA reduction was noted between agents: no sign of angiographic leakage was observed throughout weeks 2 and 4 in all treatment groups (p<0.0001). By week 6, slight recurrence of leakage was noted in ranibizumab and bevacizumab groups (28% and 4% respectively). At week 8, even though there was a higher percent change in MLA for ranibizumab (52%, p<0.001) and bevacizumab (19%, p<0.0001) in comparison to baseline, the effect in inhibition of the leakage in FA were still significant. There was no sign of angiographic leakage for aflibercept until 10 weeks.

The recurrence of the leakage was detected at the distal loops of the PRNV vasculature during the drug washout. By week 10, no significant difference in MLA compared to baseline
was noted for ranibizumab (p=0.1646) but showed otherwise for aflibercept (p<0.0001) and bevacizumab (p<0.001). It was demonstrated that the 3 anti-VEGF agents were very effective in reducing angiographic leakage for at least 8 weeks in this rabbit model (Fig. 5b).

3.4. Suppression of VEGF by preventive treatment of the anti-VEGF agents can delay initial progression of PRNV development

To understand how anti-VEGF therapy as an intervention against retinal angiogenesis works, three anti VEGF agents were given via intravitreal injection to 3 naïve eyes respectively at 1 day prior to DL-AAA injection. The preventive intervention of the anti-VEGF agents effectively delayed the progression of the PRNV development. In comparison with non-pretreatment eyes, the PRNV onset was relatively delayed at least 4 to 6 weeks which started around 8 to 10 weeks after DL-AAA injection (Fig. 6b). The inhibition of the antiangiogenesis varied in timing and degree among the 3 anti-VEGF agents (Fig. 6a). These results provide support for a role for VEGF in the initial development of the PRNV, and it also suggests that VEGF is the primary initiator of the PRNV development in this DL-AAA model.

3.5. VEGF concentrations were significantly increased in vitreous and aqueous of the PRNV eyes

VEGF-A expressions in vitreous was 2.68 ± 1.88 pg/mL and aqueous was 12.68 ± 2.73 pg/mL (mean ± SD, n = 4) from the naive eyes of the Dutch-Belted Rabbits. Increased VEGF levels were observed from all PRNV eyes (n=4). The mean VEGF-A concentration was 289.81 ± 69.64 pg/mL in vitreous and 18.25 ± 4.07 pg/mL in aqueous. VEGF-A levels in vitreous demonstrated statistically significant between PRNV and naïve eyes (p<0.0001). The VEGF-A Level in aqueous was also significantly different between the naive and the PRNV eyes (p = 0.0072).

4. Discussion

Retinal and choroidal vascular diseases such as nvAMD, DME, RVO, and PDR are major causes of blindness globally (Wong et al., 2014; Yau et al., 2012). The discovery that VEGF is a key pathogenic factor in these diseases along with intravitreal administration of anti-VEGF agents has provided an important breakthrough in the treatment. However, a key unmet need in the current management of these conditions is the frequent recurrence as soon as the effect of an intravitreal injection wears off. This leads to the need for frequent retreatments. Development of treatments with longer duration is urgently needed. To facilitate this, animal models of retinal angiogenic diseases provide an intermediate step between in vivo experimentation and human clinical trials. However, currently used animal models of CNV or RNV are limited because the CNV tends to regress within a few weeks and does not recapitulate the condition in human. In non-human primate (NHP) laser-induced CNV models, intravitreal anti-VEGF injections almost eliminate vascular leakage (Husain et al., 2005; Nork et al., 2011). However, leakage often recurs in human eyes but not in NHP. Nevertheless, enhanced experimental CNV leakage was induced in rabbits by intraocular implants containing both VEGF and bFGF for 2-4 weeks as a preclinical model (Wong et al., 2001; Wong et al., 2017). In this study, we developed a novel rabbit model of persistent leakage from PRNV using intravitreal injection of DL-AAA. The mechanism by which DL-AAA causes retinal disruption and PRNV development are not well understood. Vascular leakage, inflammation, and retinal ischemia due to retinal disruption are likely to promote the RNV as suggested by previous studies (Penn JS, 2008; Sapieha P and Lachapelle P, 2010). Ischemia
inarguably precedes the development of neovascularization in the retina as well as in other tissues. In the early stage (within 2 weeks) of this study, ischemia may play more important role than the late stage, because of the leakage were observed in existing vessels (Takahashi T, 1999). After 4 weeks, however, the leakage was observed only from the new tuft of PRNV in visual streak area. Therefore this interesting rabbit model can provide an alternative testing avenue since experimental animal models all have drawbacks.

DL-AAA is considered to be a glia-specific toxin in the central nervous system. Glial cells of the retina include the resident immune cells, microglial cells, and two types of macroglial cell, the astrocyte and the retina-specific Mueller-glial cell (Bringmann et al., 2006). Müller cells are the most predominant glial element in retina. As an important bridge connecting nerves and blood vessels in the retina, Müller cells participate in forming and maintaining the blood–retinal barrier. The external limiting membrane (ELM) of the retina is formed from junctions between Müller cells and photoreceptor cell inner segments. The ELM plays a key role in maintaining retinal structure by providing both mechanical strength and a physical barrier to photoreceptor integration (Lewis and Fisher, 2000; Omri et al., 2010; West et al., 2008). In pathological conditions, Müller cell-derived VEGF is a significant contributor to retinal neovascularization (Bai et al., 2009; Illes and Verkhratsky, 2016; Shen et al., 2012).

In rabbits, a single intravitreal injection of DL-AAA irreversibly disrupts the ELM to an extent whereby photoreceptors drop out of the outer nuclear layer to reside amongst outer segments in the subretinal space (Ishikawa and Mine, 1983). The rabbit eye has several anatomical peculiarities that differentiate it from primate and rodent models. Different to the primate, rabbits do not have a macula. The visual streak is the central area of the rabbit retina, in which the densities of ganglion cell and photoreceptors are higher than elsewhere in the retina (Famiglietti and Sharpe, 1995; Vaney et al., 1991). In this study, intravitreal injection of DL-AAA resulted to major damage in the visual streak as observed in OCT and based on the histological examination of the PRNV eyes. However, the retinal damage would not be the only factor contributing to the PRNV because 15% (10/65) of the DL-AAA injected eyes were with retinal damage demonstrated in OCT but showed no retinal leakage and without PRNV development (Fig.7).

We propose that Müller glial activation and dysfunction is the key player in the PRNV development of the DL-AAA rabbits. Furthermore, the effectiveness of anti-VEGF in preventing against the progression of PRNV formation in the DL-AAA eyes suggests that retinal Müller cell-derived VEGF is a major contributor to DL-AAA-induced retinal neovascularization. Selective ablation of Müller cells led to an imbalance between VEGF-A and PEDF and impaired retinal function due to photoreceptor apoptosis, blood-retinal barrier breakdown and intraretinal neovascularization (Shen et al., 2012). We also observed increased GFAP expression in this DL-AAA rabbit model also suggests microglia activation. GFAP is an intermediate filament (IF) protein that is expressed by numerous cell types including Müller glia in retina. The spread of GFAP-positive astrocytes was demonstrated where Müller glia was constitutively active in this model. All these pathological changes resembled those observed evidence of diabetic conditions promote increased glial cell activation in animal and clinical studies (Mizutani et al., 1998; Rungger-Brandle et al., 2000).
We are aware of previous work by other groups using DL-AAA which described vascular tel-angiectasis and increased vascular permeability in rats (Shen et al., 2010) but failed to elimi-nate Müller cells in monkeys (Bringmann et al., 2006; Mizutani et al., 1998; Rungger-Brandle et al., 2000; Shen et al., 2011). The anatomical differences between species may explain the differences described. A major difference between rabbit and primate eyes is that the retinal vascular pattern in rabbit is merangiopic. The retina blood vessels extend medial and lateral to the optic disc. Most of the retina is avascular and the blood supply is derived from the choriocapillaris (De Schaepdrijver et al., 1989; Duijm et al., 1996). A key advantage of this newly-developed rabbit PRNV model is the persistence of leakage which enables it to be used to examine the efficacy of new drugs for sustained therapeutic effect. Another key advantage of this rabbit model is the significantly lower cost compared to non-human pri-mates, and yet the rabbit eye is large enough to accommodate drug administration into the vitreous. Further advantage of the rabbit model is the fact that any microvessel growth occurring away from the medullary rays must be due to neovascularization as rabbit retina is avascular (Famiglietti and Sharpe, 1995). More importantly, all these pathological changes and PRNV will build up over 48 weeks and with no regression and without treatments. These features make the rabbit an important model for the study of retinal angiogenic diseases. Therefore, this model provides a unique tool to evaluate novel anti-VEGF formulations and therapies with respect to their duration of action in comparison to the currently used drugs.

The role of VEGF-A in mediating the leakage observed is supported by the demonstration of markedly elevated VEGF levels in the vitreous (289.8 pg/mL) and the aqueous (18.3 pg/mL) of the PRNV eyes (n=4) at 8 weeks after the DL-AAA induction. It was almost 41 folds higher than the naïve eyes in vitreous (2.7 pg/mL) and 1 fold in aqueous (12.7 pg/mL). We have also demonstrated that intravitreal administration of anti-VEGF agents effectively inhibited PRNV leakage for 8 to 10 weeks, which further supports the important role of VEGF in mediating the leakage observed in this rabbit model.

This work is the first to demonstrate that intravitreally injected anti-human-VEGF agents can exert an effect on retinal neovascularization in preclinical rabbit model. There were no specific non-clinical studies conducted to examine mechanism and pharmacodynamic differences with the 3 anti-human-VEGF agents following intravitreal administration in rabbits. Similar binding affinities with human VEGF and PIGF isoforms were found from non-human species such as the mouse, rat and rabbit for aflibercept but not, or not to a similar degree for ranibizumab and bevacizumab. As a result, aflibercept has been found to effectively inhibit pathological vascular leak in all animal models of ocular neovascular disease and vascular leak tested to date (EMA, 2012). Although bevacizumab and ranibizumab are also effective against rabbit VEGF, their cross-reactivity with rabbit VEGF are much weaker than in humans. The affinity binding to rabbit VEGF-A is about 40-fold less than that to human VEGF-A for ranibizumab and 10-fold less for bevacizumab (AusPAR, 2011; van der Flier et al., 2005). Due to the differences in binding affinity, one limitation for this rabbit model is that ranibizumab and bevacizumab may have a shorter pharmacodynamic effect in rabbits than in human.
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References


Figures

Figure 1. PRNV development after single DL-AAA intravitreal injection in a Dutch Belted rabbit. (A) Fundus photographs (FP) and FA were taken from a representative PRNV eye for over 48 weeks. Pre-PRNV show the normal retinal profile in both FP and FA with medullary ray, optic nerve head (ONH) and retinal vasculature. The process of new blood vessel formation from existing vessel networks and hyperfluorescent areas in the visual streak of the retina is shown from 2 to 48 weeks after DL-AAA injection. (B) Early and late phase FA images show the filling of the capillary tufts in the PRNV lesions with the development of circumferential hyperfluorescence happening in the later phase of the angiogram. There is rapid increase in PRNV lesions for the first 4 weeks which stabilized after 8 weeks. No change in PRNV lesions was observed in FA from 8 to 16 weeks.
Figure 2. SD-OCT in early development of PRNV. Two-Dimensional cross-sectional SD-OCT images of PRNV induced in a PRNV rabbit retina. Image extracted approximately 5mm distance downwards from ONH of the rabbit retina over 8-weeks period. Hrs, hours; W, week.
Figure 3. Light photomicrographs of H&E and GFAP staining. Representative light photomicrographs of H&E and GFAP stained paraffin sections from a DL-AAA-treated eye (A, B, C, D, E and F) and a vehicle-treated eye (G and H) at 24 weeks. In DL-AAA-treated eye, two retinal regions are compared between central retina (A, B, C and F) and peripheral retina (D and E) from the same histologic section (A) across the central to peripheral retina. 
(A) A region of central retina showing complete loss of photoreceptors and disruption of the inner and outer nuclear layers in the vertical plane below the optic nerve head in DL-AAA-treated eye. (B) Higher magnification in same area (arrows) showing the new vessels grow in surface of the disruptive retina. (C) Regional of central retina showing both inner nuclear layers and the outer nuclear layer are markedly attenuated, no ganglion cell layer was found. (D) Region of peripheral retina showing the retinal structure mainly remains intact in comparison to the vehicle-treated eye (G). In vehicle-treated eye, some Müller cells exhibit faint GFAP immunoreactivity (H), while more pronounced staining of Müller cells is evident in DL-AAA-treated retina (E and F; brown). The most intense GFAP immunoreactivity and disruption of the Müller glial cells was observed in the visual streak area (central area) of DL-AAA-injured retina (F).
Figure 4. Concentration of VEGF-A in vitreous and aqueous. Vitreous (A) & Aqueous (B) samples analysis of VEGF-A levels by ELISA from duplicates of Naïve (n=4) and PRNV (n=4) respectively. Eyes were harvested 8 weeks post DL-AAA intravitreal injection. Data is displayed as mean ± SD. Data analyzed with t-test based on the absolute value. ** denotes significance (p < 0.01); **** denotes significance (p<0.0001). Statistical significance: (p ≤ 0.05); PRNV: Persistent Retinal Neovascularization; Control: Contralateral Naïve eyes.
Figure 5. Late phase FA and leak measurement in anti-VEGF therapy. (a) The inhibition of vascular permeability in PRNV area is shown in late phase FA from three representative subjects after the anti-VEGF therapy. Each subject in the rows were treated with Drug A (Aflibercept), B (Ranibizumab) and C (Bevacizumab). The columns show the time points from left to right after the treatments of the Anti-VEGF agents. W, week. (b) Graphical representation by percentage change of mean leak areas (MLA) after the Anti-VEGF therapy. Mean Leak Area was normalized as % of baseline over a 10-weeks’ time-point. Data is displayed as Mean ± SD and analyzed with Two-way ANOVA. Pre denotes Pre-Anti-VEGF; W, weeks. Statistical significance: ns, no significance; **** p<0.0001; *** p<0.001; p ≤ 0.05 as statistically significant.
Figure 6. Suppression of VEGF in prevention of PRNV development. (a) Late phase FA were taken from 3 PRNV eyes that received the pretreatments, A, Aflibercept, B, Ranibizumab, C, Bevacizumab, respectively. The 3 anti-VEGF agents were given by intravitreal injection one day before the DL-AAA. The columns from left to right are the time points of PRNV development over 18 weeks. Naïve denotes baseline before the treatments of anti-VEGF agents and DL-AAA. PRNV-W denotes PRNV timing in weeks after the DL-AAA injection. (b) Graphical representation in Pixels for PRNV eyes that received the drugs A, B and C. Naïve denotes baseline before the treatments of anti-VEGF agents and DL-AAA. PRNV-W denotes PRNV timing in weeks after the DL-AAA injection.
Figure 7. Retinal Damage without PRNV development. Representative Fundus (A), FA (B) and OCT (C, D) image from DL-AAA-treated eye over 48 weeks. The images of A, B and C show retinal damage with no signs of PRNV development and leakage in FA (B). Blue circle line represents the area of retinal damage and Yellow line (C) indicates the section of the OCT scan (D).
Figure 8. Measurement of Leak area. Leak area analysis using open-source software (ImageJ). (A) FA shows areas of late hyperfluorescence from the retinal neovascularization. Image analysis of the leak area was based on the brightness of the optic disc blood vessels. Vascular leakage area was evaluated by firstly obtaining the (B) leakage and blood vessels area and then subtracting the image by the vessels area to obtain the leak area (C).
Highlights

- None of the animal models of retinal neovascularization used for drug development can satisfactorily recapitulate the recurrent nature of retinal neovascular disease in human.
- A novel persistent retinal neovascularization (PRNV) rabbit model using intravitreal injection of DL-AAA, characterized by persist leakage up to 48 weeks.
- VEGF plays a significant role in mediating the leakage observed in PRNV model.
- Intravitreal aflibercept, ranibizumab and bevacizumab all are very effective in reducing angiographic leakage in PRNV model.
- Aflibercept demonstrated a longer duration of action compared with the others.
- This model is suitable to evaluate novel therapies against VEGF and in particular, to compare their duration of action.