Effect of VEGF Trap on Normal Retinal Vascular Development and Oxygen-Induced Retinopathy in the Dog

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PURPOSE. To evaluate the effects of a vascular endothelial growth factor trap (VEGF Trap) on retinal vascular development and pathologic neovascularization (NV) in the canine model of oxygen-induced retinopathy (OIR).

METHODS. Newborn dogs (postnatal day [P]1) were exposed to 100% O₂ and then returned to room air on P5. VEGF Trap (5, 25, or 250 µg) was injected intravitreally in one eye and human FC (hFc) injected in the fellow eye of air control and oxygen-treated dogs on P8. The retinal vasculature and NV were evaluated on P21. Other oxygen-exposed animals received 5 µg of VEGF Trap or hFc on P22 after confirmation of retinopathy of prematurity (ROP)-like pathology and were evaluated at P45.

RESULTS. In air controls, both the vascularized area of the retina and the density of superficial capillaries were reduced in 250 or 25 µg VEGF Trap-injected eyes, and deep capillaries were absent. Eyes that received the 5 µg dose were indistinguishable from controls. In oxygen-treated animals, all eyes injected with VEGF Trap exhibited markedly less intravitreal NV than that of hFc-injected fellow eyes, irrespective of dose. Retinal vascular area in OIR animals was significantly reduced in eyes injected with 250 or 25 µg of VEGF Trap, but the 5 µg dose did not inhibit retinal revascularization. Eyes with existing NV that received 5 µg VEGF Trap at P22 exhibited substantial resolution of OIR pathology at P45.

CONCLUSIONS. The VEGF Trap inhibited the formation of NV, but higher doses also inhibited revascularization of retina when injected at P8. In contrast, the lowest dose tested effectively blocked NV and caused regression of existing NV, without appreciably affecting vasculogenesis or retinal revascularization. These findings suggest that dose selection is an important variable when considering the use of VEGF-targeting agents for the treatment of ROP. (*Invest Ophthalmol Vis Sci.* 2010;51:4039–4047) DOI:10.1167/iov.10-6798

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The primary or superficial retinal vasculature in neonatal dogs and fetal humans forms centrifugally by vasculogenesis, de novo formation of blood vessels by differentiation, and coalescence of vascular precursors or angioblasts.^{1,2} Angioblasts expressing CXCR4 and CD39 differentiate and migrate through cell-free spaces created by Muller cell processes, assemble into cords, and then primordial capillaries.^{1,2} During formation of the dog primary retinal vasculature, vasoproliferative activity is low and most of the cells in mitosis are abluminal in position and appear to be astrocytes, supporting the view that primary retinal vessel assemblage occurs initially by the process of vasculogenesis.^{1–3} The secondary or deep capillary network develops by angiogenesis, proliferation, and migration of endothelial cells from previously formed superficial retinal blood vessels.

Human retinopathy of prematurity (ROP) is the major cause of blindness in children. Infants born prematurely have incompletely vascularized retinas because the peripheral retina is avascular. ROP occurs with oxidative stress, including exposure of the developing retinal vasculature to hyperoxia. The infants with the most immature retinal vasculature have the greatest risk of ROP.⁴ The accepted therapies for ROP are ablation of the peripheral avascular retina, the source of angiogenic growth factors, with cryotherapy or laser.^{4,5} Although these therapies are sometimes effective, a new therapeutic approach is desirable not only to improve control of neovascularization (NV) but also because peripheral retina is destroyed by these ablative therapies.

Although an ROP-like retinopathy can be induced in many species by exposing neonatal animals to hyperoxia, the vasculopathy in dog most closely resembles that seen in human ROP.^{6,7} Exposure of 1-day-old dogs to hyperoxia for 4 days results in cessation of vasculogenesis as well as vaso-obliteration or destruction of portions of the developing retinal vasculature.⁸ When the animals are returned to room air, the vasoproliferative phase of oxygen-induced retinopathy (OIR) is initiated in response to the relative hypoxic state of the poorly vascularized inner retina.⁸ Three days after return to room air (postnatal day [P]8), there is extensive proliferation of cells in the retinal vasculature, suggesting that subsequent revascularization in the dog model of ROP occurs principally by angiogenesis.³ By P21, dilated and tortuous retinal vessels are present in the posterior pole, vascularization of peripheral retina remains incomplete, and vitreous hemorrhage and florid intravitreal (ITV) NV are present, which may be accompanied by ITV hemorrhage.⁷ At P45, persistent ITV NV causes tractional retinal folds, tented ITV vascularized membranes, and severe vitreous synechysis in canine OIR eyes. Immunohistochemical analysis revealed inner retinal astrogliosis occurs at the edge of the vasculature and in avascular retina.⁹ These results demonstrate that end-stage OIR in dog shares many features with chronic human ROP.⁷

TABLE 1. Numbers of Subjects, Doses, and Times of Injection

	Day Injected	Trap and FC Dose		
		5 μ g	25 μ g	250 μ g
Air controls	P8	3	3	3
Oxygen treated	P8	4	4	3
Oxygen treated	P22	3		

It is now apparent that vascular endothelial growth factor (VEGF) plays a critical role both in retinal vascular development^{10,11} and in pathologic angiogenesis in ischemic retinopathies^{12–14} and other forms of ocular NV.¹⁵ During development of the superficial vascular network in rat, VEGF is produced by astrocytes in advance of the centrifugal expansion of the vasculature to ora serrata.¹¹ However, in human and dog, the astrocytes do not precede the forming inner vasculature.^{9,16} VEGF production in inner retina has been observed in humans but the cells producing it were not defined.¹⁰ During formation of the deep capillary network, Muller cell production of VEGF is increased in response to the physiologic hypoxia induced by neuronal maturation.¹⁷ Neutralizing VEGF inhibits superficial retinal vasculature development,¹⁸ perhaps by inhibiting endothelial cell migration and the formation of filopodia used for guidance in forming the retinal vasculature.¹⁹

Similarly, when the retinal vasculature is attenuated by occlusive events such as diabetic or sickle cell retinopathy, hypoxia stimulates VEGF expression, resulting in pathologic NV in the retina and at the interface of vitreous and retina.^{19–21} Elevated levels of VEGF have been found in both human ROP²² and animal OIR models.²³

Recently, reagents targeting VEGF have been developed and have been approved for the treatment of the neovascular or “wet” form of age-related macular degeneration, whereas others are in late-stage clinical trials for this indication.^{24–26} Results to date have been quite promising and the side effects appear limited^{24–26} at least in part because, in contrast to developing blood vessels, the blood vessels of the adult retina and choroid are fully differentiated²⁷ and maintenance of the structural and functional integrity of the vasculature in the adult eye may no longer be dependent on VEGF.

Experimental interventions that block VEGF signaling have been shown to inhibit formation of ITV NV in rodent OIR models.^{28–30} However, the extent to which these interven-

FIGURE 2. Mean area ($\pm$ SD) of retina vascularized in air control animals treated with hFc (black bars) or VEGF Trap (open bars). The overall effect of treatment on retinal vascular area was statistically significant by ANOVA ($F = 21.786$; $P = 0.003$), as was the treatment $\times$ dose interaction ($F = 7.984$; $P = 0.02$). The difference in vascular area between the VEGF Trap-treated and hFc control eyes was significant at the 250 μ g ($*P = 0.01$) and 25 μ g ($**P = 0.05$) doses (Tukey's test).

tions might also inhibit retinal revascularization after vaso-obliteration has not been resolved.³¹ Nevertheless, several uncontrolled clinical studies have been conducted on premature infants with stage 4 and stage 5 ROP using ITV administration of bevacizumab, an antibody that binds and neutralizes all VEGF-A isoforms. In these limited studies, not only was neovascular regression observed but also regression of the hyaloid vasculature in vitreous.^{32,33}

The purpose of this study was to evaluate one of the VEGF-targeted reagents presently being tested in clinical trials, the VEGF Trap, in the canine model of OIR. The VEGF Trap blocks all isoforms of VEGF-A, as well as the related molecule, placental growth factor (PlGF). This study demonstrates that selective inhibition of pathologic ITV NV can be achieved at doses that do not appreciably affect normal retinal vasculogenesis or revascularization of the retina after hyperoxic insult.

FIGURE 1. ADPase-incubated retinas from air-reared 21-day-old dogs. One eye from each animal received 50 μ L of human Fc and the other eye 50 μ L of VEGF Trap. VEGF Trap at 25 and 250 μ g caused reduced capillary density, enlarged capillary-free zones around arteries (*), and constriction of vasculature, whereas the 5 μ g dose of Trap had no apparent effect. The 250 μ g dose also caused aneurysmal-like formations (arrows). Scale bar, 0.5 mm.

METHODS

Litters of 1-day-old purebred Beagle pups and nursing mothers were provided by Covance (Cumberland, VA). Animals were handled in accordance with the ARVO guidelines for humane use of laboratory animals for eye research, as reported previously.^{3,7,8} Litters of six 1-day-old animals were divided as follows: (1) four or five animals were exposed to 100% oxygen for 4 days; and (2) one or two littermates were kept as room air controls (Table 1). The nursing mother spent 12 hours per day with each group of puppies. Post hyperoxia, animals were returned to room air on P5. On P8, 50 μ L of a solution containing VEGF Trap (5, 25, 250 μ g) was injected in one eye. The fellow eye received the same dose of a control protein comprising the constant domain of human immunoglobulin G, subclass 1 (human Fc [hFc]). The effect of VEGF Trap on normal vasculogenesis and angiogenesis was similarly evaluated in air-raised littermates. Animals were humanely euthanatized at P21, during the active vasoproliferative stage in the OIR groups, by an intraperitoneal overdose of sodium pentobarbital.

In another experiment, the effect of VEGF Trap or hFc (5 μ g in 50 μ L) on established NV was evaluated. After exposure to hyperoxia and a 17-day period of recovery in room air, eyes were injected with VEGF Trap or hFc at P22 after the presence of NV was confirmed by fundus photography. This group of animals was maintained until P45.

ITV Injection

After the induction of general anesthesia by fluothane inhalation and intubation, a shallow canthotomy was performed to enlarge the surgical field. A 30-gauge needle was introduced at a 60° angle to the iris and the needle was advanced 5 mm through the pars plana to avoid touching the hyaloid vasculature. A 50 μ L bolus of VEGF Trap or hFc was injected into vitreous and the needle was slowly withdrawn after intraocular pressure had normalized, as evidenced by loss of postinjection pallor. The canthotomy was sutured and eyes were treated daily for 5 days with triple antibiotic ointment (AK Poly-Bac; Akorn, Buffalo Grove, IL).

Tissue Processing

Animals were euthanatized by an overdose of sodium pentobarbital at P21 or P45. A blood sample (2 mL) was drawn intracardially for plasma analysis of active and bound VEGF Trap before cardiac arrest, as previously described.³⁴ Eyes were enucleated, fixed for 7 minutes in 25% Karnovsky's fixative at room temperature, and washed several times in cold 0.1 M sodium cacodylate buffer with 5% sucrose. The anterior segments were removed and the vitreous body was removed in toto with forceps. Retinas were dissected from the retinal pigment epithelium and both vitreous and retina were fixed for 20 hours in 2% paraformaldehyde/0.1 M sodium cacodylate buffer at 4°C. After several washes in cold 0.1 M sodium cacodylate buffer containing 5% sucrose at 4°C, the tissues were incubated for ADPase activity, as previously

FIGURE 3. (A–F) Representative micrographs of peripheral superficial retinal vasculature in ADPase-incubated retinas at P21. The retinal vasculature appeared less dense in eyes receiving 250 and 25 μ g VEGF Trap (D, E) compared with hFc-injected fellow eyes (A, B). The 5 μ g VEGF Trap-injected eye (F) appeared similar to the fellow eye injected with 5 μ g hFc (C). (G) Mean percent vascular area or vascular density ($\pm$ SEM) of the superficial plexus expressed as percentage vascular area at P21. The overall effect of treatment on retinal vascular density was statistically significant by ANOVA ($F = 140.5$; $P < 0.0001$) and treatment $\times$ dose interaction was also significant ($F = 18.62$; $P = 0.0002$). The difference in vascular density was significant in eyes injected with 250 and 25 μ g ($*P < 0.01$; Tukey's test). In contrast, injection of 5 μ g VEGF Trap did not result in a reduction of capillary density.

described to label blood vessels and angioblasts.³⁵ Retinas and vitreous bodies (containing ITV NV in OIR animals) were incubated for ADPase enzyme histochemical activity and then flat-embedded for image analysis and subsequent sectioning.³⁵

Flat-mounted retinas were imaged under darkfield illumination using a digital camera (MicroPublisher; QImaging, Surrey, BC, Canada), mounted on a stereomicroscope (Stemi 2000; Carl Zeiss MicroImaging), and digital image and photo-editing software (Adobe Photoshop, version 7) on a desktop computer (Macintosh G4). The vitreous body was also imaged. With darkfield illumination, the lead phosphate reaction product, which is confined to the vasculature, appears white against a black background. A stage micrometer was photographed at the same magnifications to calibrate the tissue images for area measurements. After imaging, identical regions of retina were trimmed from the whole mount, and flat-fixed and flat-embedded in JB-4 (glycol methacrylate) as reported previously.³⁵ Sections were cut at 2.5 μ m and treated with ammonium sulfide to develop the ADPase reaction product to a brown reaction product and counterstained with thionin. Some sections were stained with periodic acid-Schiff (PAS) reagent and hematoxylin. This allowed us to examine both the pattern and radial growth of the retinal vessels in flat mounts, as well as the structure of the blood vessels and the neural retina in cross-sections using the same specimens.

Data Analysis

For area measurements, images were opened using photo-editing software (Adobe Photoshop), reduced in size to 640 $\times$ 480 pixels, and converted from red, green, blue (RGB) to grayscale. Images were saved as TIFF files and imported into NIH Image (version 1.47) for calibration (micrometers). The tracing tool was used to outline the vascularized area of retina. The mean retinal vascular area $\pm$ SD was then calculated for each treatment and control group. The area of NV in vitreous was calculated in the same way in the eyes of animals exposed to hyperoxia. The density of the retinal vasculature (percentage vascular area) was determined by capturing images from three fields of capillaries, making the images binary, and using ImageJ to compute the number of black pixels and white pixels (blood vessels) and the percentage white $\pm$ SD (SEM) as reported previously using the calculated black to white ratio macro.³⁵

The effects of drug treatment and dose on retinal vessel area and capillary density were evaluated by mixed-factorial ANOVA followed by post hoc tests. For eyes injected with VEGF Trap or hFc (5 μ g) on P22, the effects of treatment on retinal vascular area and NV were evaluated by paired *t*-test. The numbers of eyes evaluated for each treatment condition in each experiment are shown in Table 1.

RESULTS

All dogs that received ITV injections of VEGF Trap and hFc continued to grow normally and appeared healthy. Blood samples were subjected to ELISA analysis to determine circulating levels of both free VEGF Trap (active drug, not bound to VEGF) and bound VEGF Trap (VEGF Trap that has already bound VEGF, creating a stable, inactive complex).³⁴ Irrespective of dose, free VEGF Trap was below detectable levels in all animals (lower limit of quantification [LLQ] 20.56 ng/mL). Bound, inactive VEGF Trap also was below detectable levels (LLQ 4.17 ng/mL) in all animals that received ITV injections of 5 or 25 μ g, whereas very low levels of bound VEGF Trap were detected in animals that received 250 μ g of VEGF Trap (30.68–53.03 ng/mL, mean = 39.09 ng/mL). Taken together, these data indicate that systemic exposure was minimal after ITV injection of VEGF Trap.

Effects of VEGF Inhibition on Retinal Vascular Development

In air-reared dogs, the superficial capillary network in ADPase-stained retinal flat mounts exhibited capillary-free zones around arteries that were clearly enlarged at the 250 and 25 μ g VEGF Trap doses (Fig. 1). Moreover, the retinal vascular area was significantly reduced in eyes treated with VEGF Trap at the 250 and 25 μ g doses compared with the fellow, control eye treated with hFc (Fig. 2). Capillary density in the superficial plexus was also reduced (Fig. 3), and development of the deep capillary plexus was inhibited in eyes that received 250 or 25 μ g VEGF Trap (Fig. 4). In contrast, ITV administration of 5 μ g VEGF Trap had no detectable effect on retinal vascular area or

FIGURE 4. JB-4 sections of ADPase-incubated retinas from air-reared animals shown in Figure 1 that were incubated with ammonium sulfide to develop the ADPase. The secondary or deep capillary network (*arrow-heads*) was present in the hFc retinas and the retina treated with 5 μ g of Trap, whereas the 250 μ g of Trap retina lacks a deep capillary network. The 25 μ g dose also lacked a secondary capillary network (results not shown). (Black ADPase reaction product and thionin counterstain). Scale bar, 20 μ m.

morphology (Figs. 1, 2), the capillary density in the superficial vascular plexus (Fig. 3), or the development of the deep capillary network (Fig. 4).

Effects of VEGF Trap on Pathologic NV and Revascularization in OIR

Animals were exposed to hyperoxia at P1 and returned to room air 4 days later. On P8, each animal received a 50 μ L injection of VEGF Trap (5, 25, or 250 μ g) in one eye and an equal amount of hFc in the fellow eye (Table 1). At P21, the retinas and vitreous bodies were removed and incubated for ADPase activity. The retinal vascular area at P21 was significantly reduced in animals exposed to hyperoxia (see Fig. 5, 6) compared with air-reared controls (Fig. 2), irrespective of drug treatment. The area of the retina covered by the superficial vascular plexus was significantly smaller in the eyes receiving 250 ($P < 0.01$) or 25 μ g ($P < 0.05$) VEGF Trap compared with the Fc-injected fellow eyes (Figs. 5A, 5B, 5D, 5E). However, the retinal vascular area in eyes receiving 5 μ g VEGF Trap was indistinguishable from the hFc-injected fellow eyes (Figs. 5C, 5F).

All animals exposed to hyperoxia developed pathologic NV. Representative examples of the ADPase-incubated vitreous bodies from animals in each group are shown in Figures 5G–L. ITV NV was significantly smaller in VEGF Trap-treated eyes (0.33 ± 0.35 mm²) compared with Fc-treated control eyes (6.88 ± 7.44 mm²), across all doses administered ($P = 0.032$). The mean areas of ITV NV for each group are shown in Figure 6B. VEGF Trap at any dose almost completely inhibited formation

of ITV NV (Figs. 5G–L and 6B). However, post hoc analysis showed that this difference was statistically significant only for the 5 and 25 μ g doses. That the difference for the 250 μ g dose was not significant is attributable to the fact that the severity of the NV in the hFc-treated fellow eyes was markedly less severe than that for the two lower-dose levels, which was perhaps a result of litter-to-litter and animal-to-animal variability. The retinas of 250 μ g Fc and VEGF Trap eyes appeared histologically similar (Fig. 7), suggesting the Trap was not toxic.

To evaluate the effects of VEGF Trap on established ITV NV, three additional animals were exposed to hyperoxia, as described earlier. By P21 all three animals exhibited OIR as determined by fundus photography, including extensive ITV neovascular membranes and hemorrhage (examples of eyes from one animal are shown in Figs. 8A, 8B). At P22, one eye of each animal received 5 μ g of VEGF Trap and the fellow eye 5 μ g of hFc. At P45, the eyes were rephotographed (Figs. 8C, 8D) and then the animals were euthanatized and the eyes taken for histopathologic analysis of the retinal and ITV vasculature, as described earlier. At P45, the ITV membranes appeared resolved by fundus photography in the VEGF Trap-injected eyes (Fig. 8D). The area of ITV NV was still substantial at P45 in all Fc-injected eyes (Figs. 9C and 10B), but little or no NV remained in the eyes injected with 5 μ g of Trap (Figs. 9D and 10B, $P < 0.05$ compared with the hFc-treated control eyes). When flat mounts of the ADPase-incubated retinas were analyzed, the area of the retinal vasculature was greater in two of the three eyes that re-

FIGURE 5. (A–F) ADPase-incubated retinas from P21 oxygen-exposed dogs that were treated with hFc or VEGF Trap. The 250 μ g and 25 μ g doses of VEGF Trap inhibited centripetal growth of blood vessels toward ora serrata in a dose-dependent manner (D, E) compared with the hFc-treated eyes (A, B). In contrast, the retinal vascular area in oxygen-exposed eyes treated with 5 μ g VEGF Trap (F) was greater than that in the hFc-injected control eyes (C). Moreover, capillary density was not appreciably reduced. Scale bar, 1 mm. (G–L) ADPase-incubated vitreous bodies from oxygen-treated eyes injected with 250, 25, or 5 μ g hFc or VEGF Trap. The hFc-injected eyes had considerable intravitreal (ITV) neovascularization (NV; paired arrows in G–I), whereas the vitreous bodies from VEGF Trap-treated eyes had no appreciable ADPase-positive vitreous NV at all doses tested (arrows represent optic nerve head in J–L). Scale bar, 2 mm.

FIGURE 6. (A) The mean retinal vascular areas ($\pm$ SD) of P21 oxygen-exposed animals was smallest in the 25 and 250 μ g Trap-treated eyes compared with hFc-injected eyes (* $P < 0.05$, Tukey's test). The overall effect of treatment on retinal vascular area was statistically significant by ANOVA ($F = 7.953$; $P = 0.032$), as was the treatment $\times$ dose interaction ($F = 5.14$; $P = 0.037$). There was no difference in retinal vascular area between hFc control eyes and eyes that received 5 μ g VEGF Trap. (B) The mean area of ITV NV was reduced significantly in all animals treated with 25 and 5 μ g VEGF Trap compared with hFc (* $P < 0.05$; Tukey's test). The overall effect of treatment on vitreal NV also was statistically significant by ANOVA ($F = 6.696$; $P = 0.032$). Although ITV NV was suppressed to an equivalent extent in the 250 μ g dose group, this was not statistically significant due to the lower level of NV observed in the fellow eyes treated with 250 μ g hFc.

ceived VEGF Trap compared with hFc-injected fellow eyes (Fig. 10A).

DISCUSSION

This study demonstrated that single ITV injections of VEGF Trap can prevent the development of pathologic ITV NV in the canine model of OIR, and also produce regression of established neovascular membranes. These effects were observed at all doses of VEGF Trap tested: 250, 25, and 5 μ g/eye.

An abundance of evidence now demonstrates that VEGF plays a critical role in both normal vascular development in tissues throughout the body and pathologic NV in various disease states³⁶⁻³⁹; the present findings are in agreement with this body of literature. With respect to the retina, previously published studies have consistently reported that administration of various pharmacologic inhibitors of VEGF-A, including antibodies, aptamers, and soluble receptors, are capable of ameliorating pathologic NV in murine and rat models of OIR.^{29,30,40} Although it is also generally accepted that VEGF plays an indispensable role in vascular

FIGURE 7. JB-4 sections of oxygen-exposed retinas showed no apparent toxicity of the VEGF Trap (i.e., retinas treated with Trap and Fc look the same; hematoxylin and PAS staining). Scale bar, 20 μ m.

FIGURE 8. Fundus photographs of fellow eyes at P22 (A, B) before ITV injection, and on P45, 23 days after 5 μ g hFc (C) or VEGF trap (D). The neovascular membrane over the disc (*) persists in the hFc eye, whereas VEGF Trap reduced tortuosity of retinal vessels and the neovascular membrane (*) has regressed.

development in the retina^{29–31,40} few studies have specifically evaluated the effects of VEGF inhibitors on either retinal vascular development or revascularization after exposure to hyperoxia.

Ishida et al.³¹ reported that administration of a VEGF₁₆₄-specific aptamer inhibited only pathologic NV, whereas a VEGFR-1 fusion protein also attenuated normal retinal vascular development in mice, as well as retinal revascularization in the OIR model. Similarly, we have previously reported that ITV administration of an antibody against VEGFR-2 (cp1c11) inhibited retinal vascular development as well as NV in the canine OIR model.⁴¹ However, Geisen et al.²⁹ reported that an anti-

VEGF antibody did not appear to interfere with normative revascularization of the retina post hyperoxia in the rat OIR model. Budd et al.⁴² recently observed the same result with an inhibitor of VEGFR-2 signaling (SU5416). Although the reasons for these seemingly discrepant results are presently unclear, it is important to note that the relative potency of the above-cited agents is unknown, and the effects of varying the dose were not explored.

In the present study, we evaluated the effects of VEGF Trap on normal retinal vascular development and revascularization over a 50-fold range of doses, from 5 to 250 μ g/eye. In contrast to the effect on pathologic NV, the effects of VEGF Trap on

FIGURE 9. ADPase-incubated retinas (A, B) and vitreous bodies (C, D) from eyes shown in Figure 8. The hFc-injected eye (A, C) had a smaller retinal vascular area and larger ITV neovascular formation (*paired arrows*) than those of the eye injected with 5 μ g of Trap (B and D). Scale bars: (A, B) 5 mm; (C, D) 2 mm.

FIGURE 10. Quantitation of retinal vascular areas (A) and ITV NV (B) in animals injected with 5 μ g of hFc or VEGF Trap at P22 and killed at P45. ITV NV was significantly reduced in VEGF Trap-treated eyes relative to hFc-treated fellow eyes ($P < 0.05$, paired t -test). The retinal vascular area was increased in the VEGF Trap-treated eye in two cases and unchanged in a third. However, this difference was not statistically significant.

both retinal vascular development and revascularization after exposure to hyperoxia, were clearly dose dependent. Specifically, in air-raised animals, the area of retina covered by the superficial vascular plexus was significantly reduced in the 250 and 25 μ g/eye doses of VEGF Trap and the development of the newly forming deep capillary plexus was also inhibited. In animals exposed to hyperoxia, the 250 and 25 μ g doses also reduced the extent of retinal revascularization in a dose-dependent fashion. However, ITV administration of 5 μ g VEGF trap did not affect any of the above-mentioned parameters, in either oxygen or air-raised pups. This dose effectively prevented the development of NV but the retinal vasculature of pups treated at this dose was indistinguishable from that of similarly raised hFc-treated controls. Thus, selective inhibition of pathologic NV in OIR may be achieved with a potent, pan-VEGF inhibitor by appropriately titrating the dose.

We have recently demonstrated that VEGFR-2 (also known as kinase insert domain receptor) is upregulated in the canine model of OIR.⁴¹ In 15-day-old oxygen-exposed animals, where both established ITV NV and immature forming NV exist, we observed higher levels of VEGFR-2 immunostaining in the immature NV, as well as the reforming retinal vasculature. This explains why at high doses the VEGF Trap and VEGFR-2 antibody block both of these angiogenic events. Other groups have demonstrated that VEGF and its receptors are elevated in oxygen-induced retinopathy models in other species.^{14,43,44}

There are several possible reasons that low doses of the VEGF Trap were able to block pathologic NV without interfering with normative retinal revascularization. For example, the drug may have been occupied by VEGF in vitreous, and was not available to bind retinal VEGF. However, the low dose of VEGF Trap did not alter normal retinal vascular development in air-raised animals, where ITV levels of VEGF were undoubtedly much lower, making this explanation seem implausible. However, VEGFR-2 immunoreactivity was far greater on preretinal forming vessels in OIR than that in vessels developing by vasculogenesis in the dog retina.⁴¹ An alternative explanation is that pathologic angiogenesis is inherently more dependent on the presence of VEGF-A than are vessels developing normally by vasculogenesis. Shih and associates⁴⁵ argue that PlGF may be the critical VEGF family member and VEGFR-1 the important receptor for development and maintenance of the superficial vasculature in mouse; however, the VEGF Trap neutralizes PlGF and all isoforms of VEGF.

Some recent nonclinical studies have suggested that VEGF also serves a neurotrophic function.^{46–48} However, evaluation of retinal function in rabbit⁴⁹ and in mouse⁵⁰ after bevacizumab injection revealed no change in electroretinography (ERG). Similarly, studies in nonhuman primates have not de-

tected any adverse effect of repeated ITV administration of VEGF Trap on the mature retinal vasculature, retinal morphology, or neural retinal function as assessed by ERG (Zimmer E, et al. *IOVS* 2006;47:ARVO E-Abstract 1751). Moreover, in the present study, there were no observable morphologic changes in the neural retina of eyes receiving VEGF Trap at any dose. Importantly, the now extensive use of pan-VEGF blockers in humans, first in carefully controlled clinical studies and subsequently in clinical practice, has not been associated with impairments in neural retina structure or function.

The canine OIR model provides a useful forum for preclinical evaluation of angiogenesis inhibitors. The resultant pathology closely resembles human ROP, in an eye approximately equivalent in size and vitreous volume to that of a 25-week gestation human. Furthermore, in the canine model of OIR, the pathologic NV persists at least until P45 and does not spontaneously regress, unlike in rodent models. We have evaluated the effects of a potent VEGF inhibitor, VEGF Trap, using this model, and determined that a single injection of as little as 5 μ g effectively prevented the development of ITV NV, and also caused regression of established NV and relief of vascular tortuosity in eyes with proliferative retinopathy. However, as expected from findings obtained with other VEGF blockers, high doses of VEGF Trap also inhibited normal retinal vascular development. These results suggest that careful dose titration of VEGF inhibitors may be necessary to produce an optimal and selective inhibition of NV in the developing retina.

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