JOURNAL OF CELLULAR PHYSIOLOGY 195:241–248 (2003)

VEGF-TRAP$_{R1R2}$ Suppresses Choroidal Neovascularization and VEGF-Induced Breakdown of the Blood–Retinal Barrier

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Vascular endothelial growth factor (VEGF) plays a central role in the development of retinal neovascularization and diabetic macular edema. There is also evidence suggesting that VEGF is an important stimulator for choroidal neovascularization. In this study, we investigated the effect of a specific inhibitor of VEGF, VEGF-TRAP$_{R1R2}$, in models for these disease processes. VEGF-TRAP$_{R1R2}$ is a fusion protein, which combines ligand binding elements taken from the extracellular domains of VEGF receptors 1 and 2 fused to the Fc portion of IgG1. Subcutaneous injections or a single intravitreous injection of VEGF-TRAP$_{R1R2}$ strongly suppressed choroidal neovascularization in mice with laser-induced rupture of Bruch's membrane. Subcutaneous injection of VEGF-TRAP$_{R1R2}$ also significantly inhibited subretinal neovascularization in transgenic mice that express VEGF in photoreceptors. In two models of VEGF-induced breakdown of the blood–retinal barrier (BRB), one in which recombinant VEGF is injected into the vitreous cavity and one in which VEGF expression is induced in the retina in transgenic mice, VEGF-TRAP$_{R1R2}$ significantly reduced breakdown of the BRB. These data confirm that VEGF is a critical stimulus for the development of choroidal neovascularization and indicate that VEGF-TRAP$_{R1R2}$ may provide a new agent for consideration for treatment of patients with choroidal neovascularization and diabetic macular edema. J. Cell. Physiol. 195: 241–248, 2003. © 2003 Wiley-Liss, Inc.

Ocular neovascularization, consisting of retinal and choroidal neovascularization, is an enormous public health problem. Retinal neovascularization occurs in ischemic retinopathies, the most prevalent of which is diabetic retinopathy, the most common cause of severe vision loss in young people in developed countries (Klein et al., 1984). Choroidal neovascularization complicates several diseases in which there are abnormalities of the Bruch's membrane/retinal pigmented epithelial (RPE) cell complex, such as age-related macular degeneration (AMD), the most common cause of severe vision loss in the elderly (The Macular Photocoagulation Study Group, 1991). While retinal and choroidal neovascularization are responsible for the vast majority of severe vision loss in Americans, diabetic macular edema is the major cause of moderate vision loss (Klein et al., 1984).

Multiple stimulatory factors may contribute to the development of retinal neovascularization, but vascular endothelial growth factor (VEGF) plays a critical role. Signaling through VEGF receptors is both necessary and sufficient for development of retinal neovascularization (Okamoto et al., 1997; Seo et al., 1999; Ozaki et al., 2000). VEGF also causes breakdown of the blood–retinal barrier (BRB) (Ozaki et al., 1997), and has been implicated in the early breakdown of the BRB that occurs in diabetes (Qaum et al., 2001). In addition, VEGF is also an important stimulus for choroidal neovascularization (Kwak et al., 2000). Therefore, antagonizing VEGF is a potentially useful strategy for several ocular diseases.

Many approaches for antagonizing VEGF are being considered. One strategy is to inject relatively large inhibitors, such as aptamers or FAb fragments of anti-VEGF antibodies directly into the eye. Phase I clinical

PAC is the George S. and Dolores Dore Eccles Professor of Ophthalmology and Neuroscience.

Contract grant sponsor: Public Health Service; Contract grant numbers: EY05951, EY12609, P30EY1765; Contract grant sponsor: Foundation Fighting Blindness; Contract grant sponsor: Research to Prevent Blindness (Lew R. Wasserman Merit Awards and unrestricted grant); Contract grant sponsor: Dr. and Mrs. William Lake.

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Received 30 August 2002; Accepted 20 November 2002

DOI: 10.1002/jcp.10246

trials testing the safety and tolerability of this approach have been completed and phase II and III trials are planned or in progress. Preliminary reports suggest that inflammation may occur following intraocular injection of antibodies or aptamers, but this has not been a severe enough problem to discontinue evaluation of these approaches (Guyer et al., 2001; Schwartz et al., 2001). This approach has some concerns, because repeated intraocular injections carry risks of retinal detachment and endophthalmitis, and may not be feasible depending upon the frequency of injections required. Another strategy is to avoid repeated intraocular injections by systemic administration of small molecule VEGF antagonists (Seo et al., 1999; Kwak et al., 2000; Ozaki et al., 2000). There is a theoretical concern that some beneficial types of angiogenesis, such as collateral formation in ischemic myocardium, may be inhibited. But there are no data to support this concern and it is equally plausible that systemic inhibition of VEGF could have many additional benefits, since angiogenesis has been implicated in tumor growth, atherosclerosis, and arthritis (for review, see Folkman, 1995). Oral administration of VEGF receptor kinase inhibitors results in dramatic suppression of retinal and choroidal neovascularization and is a very promising approach (Seo et al., 1999; Kwak et al., 2000; Ozaki et al., 2000). These agents are selective, but not specific VEGF antagonists, because it is difficult to inhibit VEGF receptor kinases without inhibiting homologous kinases such as platelet-derived growth factor (PDGF) receptor kinase and c-kit, the receptor for stem cell factor (Fabbro et al., 1999; Bold et al., 2000; Drevs et al., 2000; Wood et al., 2000). The effects of these additional activities are unknown and while they are being investigated, it is prudent to consider and test more selective VEGF inhibitors.

Soluble VEGF receptors provide a very specific way to antagonize VEGF, and several studies have demonstrated that the extracellular domain of VEGF receptor 1 (VEGF-R1) has antiangiogenic activity (Goldman et al., 1998; Kong et al., 1998; Honda et al., 2000; Shiose et al., 2000; Takayama et al., 2000; Lai et al., 2001; Mahasreshti et al., 2001; Bainbridge et al., 2002; Lai et al., 2002). A disadvantage of soluble VEGF-R1 is that it is cleared fairly rapidly. Pharmacokinetic properties can be improved by linking the ligand binding domains of VEGF receptors to the Fc portion of IgG, which slows clearance by conferring the long circulating half-life of an antibody to the chimeric molecule. A potential trade off is that the relatively large size of such constructs could limit tissue penetration from the systemic circulation, which is a particularly important consideration for treatment of ocular diseases. In this study, we have evaluated both local and systemic administration of a novel chimeric molecule, VEGF-TRAP$_{R1R2}$, which comprises portions of the extracellular domain of VEGFR-1 (flt-1) and VEGFR-2 (KDR), in models of ocular neovascularization and breakdown of the BRB.

MATERIALS AND METHODS

VEGF-TRAP$_{R1R2}$

VEGF-TRAP$_{R1R2}$ (Regeneron Pharmaceuticals, Tarrytown, NY) is a recombinant fusion protein that contains Ig domain 2 of VEGF-R1 and Ig domain 3 of VEGF-R2 fused to the Fc portion of human IgG1 (Wulff et al., 2002). VEGF-TRAP$_{R1R2}$ binds VEGF with high affinity (kD $\approx$ 1 pM) and subcutaneous injection of 25 mg/kg of VEGF-TRAP$_{R1R2}$ has been shown to effectively neutralize VEGF in mice with VEGF-secreting tumors (Wong et al., 2001). Recombinant human Fc was used as a control protein.

Treatment of mice with laser-induced choroidal neovascularization

Choroidal neovascularization was generated by modification of a previously described technique (Tobe et al., 1998b). Briefly, 4–5-week-old female C57BL/6J mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and the pupils were dilated with 1% tropicamide. Three burns of 532 nm diode laser photocoagulation (75 μm spot size, 0.1 sec duration, 120 mW) were delivered to each retina using the slit lamp delivery system of an OcuLight GL Photocoagulator (Iridex, Mountain View, CA) and a hand held cover slide as a contact lens. Burns were performed in the 9, 12, and 3 o'clock positions of the posterior pole of the retina. Production of a bubble at the time of laser, which indicates rupture of Bruch's membrane, is an important factor in obtaining CNV (Tobe et al., 1998b), so only burns in which a bubble was produced were included in the study. Mice were treated with subcutaneous injections of 25 mg/kg of VEGF-TRAP$_{R1R2}$ or Fc fragment 1 day prior to laser and on days 2, 5, 8, and 11 after laser. At 14 days after laser, the mice were euthanized, serum was collected and stored, and eyes were rapidly dissected for choroidal flat mounts or frozen in optimum cutting temperature embedding compound (OCT; Miles Diagnostics, Elkhart, IN).

Some mice were given intraocular injection of 4.92 μg of VEGF-TRAP$_{R1R2}$ in one eye and 4.92 μg Fc fragment in the other eye. Two weeks later, mice were perfused with fluorescein-labeled dextran and choroidal neovascularization was measured.

Quantitative analysis of the amount of choroidal neovascularization

The sizes of CNV lesions were measured in choroidal flat mounts (Edelman and Castro, 2000) by an investigator masked with respect to treatment group. Mice used for the flat mount technique were anesthetized and perfused with 1 ml of phosphate-buffered saline containing 50 mg/ml of fluorescein-labeled dextran (2×10^6 average mw, Sigma, St. Louis, MO) as previously described (Tobe et al., 1998a). The eyes were removed and fixed for 1 h in 10% phosphate-buffered formalin. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup. Radial cuts (4–7, average 5) were made from the edge to the equator and the eyecup was flat mounted in Aquamount with the sclera facing down. Flat mounts were examined by fluorescence microscopy on an Axioskop microscope (Zeiss, Thornwood, NY) and images were digitized using a 3 color CCD video camera (IK-TU40A, Toshiba, Tokyo, Japan) and a frame grabber. Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used to measure the total area of choroidal neovascularization associated with each burn with the operator masked with respect to treatment group. Statistical comparisons were made between the size of lesions in mice

treated with VEGF-TRAP$_{R1R2}$ versus those in mice treated with Fc fragment by two-tailed *t*-test. In addition, the average size of choroidal neovascularization in each mouse was calculated and plotted against the serum level of VEGF-TRAP$_{R1R2}$ obtained by ELISA.

In some mice, the eyes were rapidly removed and frozen in optimum cutting temperature embedding compound (OCT; Miles Diagnostics). Ten μm frozen sections were cut through entire lesions and the sections were histochemically stained with biotinylated *Griffonia simplicifolia* lectin B4 (GSA, Vector Laboratories, Burlingame, CA), which selectively binds to vascular cells. Slides were incubated in methanol/H_2O_2 for 10 min at 4°C, washed with 0.05 M Tris-buffered saline, pH 7.6 (TBS), and incubated for 30 min in 10% normal porcine serum. Slides were incubated 2 h at room temperature with biotinylated GSA and after rinsing with 0.05 M TBS, they were incubated with avidin coupled to peroxidase (Vector Laboratories) for 45 min at room temperature. The slides were developed with Histo-Mark Red (Kirkegaard and Perry, Cabin John, MD) to give a red reaction product and counter stained with Contrast Blue (Kirkegaard and Perry).

Transgenic mice with increased expression of VEGF in photoreceptors

Transgenic mice with VEGF driven by the rhodopsin promoter develop subretinal neovascularization due to expression of VEGF in photoreceptors beginning at about P7 (Okamoto et al., 1997). Hemizygous transgene-positive mice were given a subcutaneous injection of 25 mg/kg of VEGF-TRAP$_{R1R2}$ or Fc fragment at P7, P10, P13, P16, and P19. At P21, the mice were sacrificed and the amount of subretinal neovascularization was quantified as previously described (Tobe et al., 1998a). Briefly, mice were anesthetized and perfused with 1 ml of phosphate-buffered saline containing 50 mg/ml of fluorescein-labeled dextran (2×10^6 average mw, Sigma). The eyes were removed and fixed for 1 h in 10% phosphate-buffered formalin. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup, radially cut from the edge of the retina to the equator in all 4 quadrants, and flat-mounted in Aquamount with photoreceptors facing upward. The retinas were examined by fluorescence microscopy at 200x magnification, which provides a narrow depth of field so that when focusing on neovascularization on the outer surface of the retina, the remainder of the retinal vessels are out-of-focus allowing easy delineation of the neovascularization. The outer edge of the retina, which corresponds to the subretinal space in vivo, is easily identified and therefore there is standardization of focal plane from slide to slide. Images were digitized using a 3 CCD color video camera and a frame grabber. Using Image-Pro Plus software, an investigator masked with respect to treatment group delineated each of the lesions and calculated the total area of neovascularization per retina as previously described (Tobe et al., 1998a).

VEGF-induced breakdown of the BRB

Adult C57BL/6 mice were given a subcutaneous injection of 25 mg/kg of VEGF-TRAP$_{R1R2}$ or Fc and on the following day VEGF-induced breakdown of the BRB was quantified as previously reported (Derevjanik et al., 2002). Mice were anesthetized with 25 mg/kg of ketamine and 4 mg/kg of xylazine, pupils were dilated with 1% tropicamide. Intraocular injections were performed with a Harvard pump microinjection apparatus and pulled glass micropipets (Mori et al., 2001). Each micropipet was calibrated to deliver 1 μl of fluid upon depression of a foot switch. Under a dissecting microscope, the sharpened tip of a micropipet was passed through the sclera just behind the limbus into the vitreous cavity, and the foot switch was depressed injecting 1 μl of 10^{-6} M human vascular endothelial growth factor (VEGF; R&D Systems, Minneapolis, MN). Six hours later, retinal vascular permeability was measured using [^{3}H]mannitol as a tracer.

Double transgenic rho/rtTA-TRE/VEGF mice with doxycycline-inducible expression of VEGF in photoreceptors (Ohno-Matsui et al., 2002) were also used. Double transgenics were given a subcutaneous injection of 25 mg/kg of VEGF-TRAP$_{R1R2}$ or Fc fragment of IgG and on the following day they were started on 2 mg/ml of doxycycline in their drinking water. The next day they were given a second subcutaneous injection of 25 mg/kg of VEGF-TRAP$_{R1R2}$ or Fc fragment and after two days, retinal vascular permeability was measured.

Measurement of BRB breakdown using [^{3}H]mannitol as tracer

Six hours after intraocular injection of VEGF in wild type mice or 2 days after rho/rtTA-TRE/VEGF were started on doxycycline, mice were given an intraperitoneal injection of 1 μCi/gram body weight of [^{3}H]mannitol (New England Nuclear, Boston, MA). After 1 h, mice were sacrificed and eyes were removed. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup and placed within pre-weighed scintillation vials. The thoracic cavity was opened and the left superior lobe of the lung was removed and placed in another pre-weighed scintillation vial. All liquid was removed from the vials and remaining droplets were allowed to evaporate over 20 min. The vials were weighed and the tissue weights were recorded. One ml of NCSII solubilizing solution (Amersham, Chicago, IL) was added to each vial and the vials were incubated overnight in a 50°C water bath. The solubilized tissue was brought to room temperature and decolorized with 20% benzoyl peroxide in toluene in a 50°C water bath. The vials were brought to room temperature and 5 ml of Cytoscint ES (ICN, Aurora, OH) and 30 μl of glacial acetic acid were added. The vials were stored for several hours in darkness at 4°C to eliminate chemoluminescence. Radioactivity was counted with a Wallac 1409 Liquid Scintillation Counter (Gaithersburg, MD).

RESULTS

Subcutaneous injection of VEGF-TRAP$_{R1R2}$ inhibits choroidal neovascularization

Bruch's membrane was ruptured at 3 locations in each eye by laser photocoagulation in C57BL/6 mice. One day prior to laser and on days 2, 5, 8, and 11 after laser, mice received subcutaneous injection of 25 mg/kg of VEGF-TRAP$_{R1R2}$ or Fc fragment. Retinal whole mounts from fluorescein dextran-perfused mice treated with VEGF-

$TRAP_{R1R2}$ (Fig. 1A,B) had areas of neovascularization that were much smaller than those seen in mice treated with Fc fragment (Fig. 1C,D). Sections through Bruch's membrane rupture sites in other mice treated with VEGF-$TRAP_{R1R2}$ showed complete or near-complete inhibition of choroidal neovascularization (Fig. 1E,F). Mice treated with Fc fragment (Fig. 1G,H) had choroidal neovascularization similar to that seen in mice treated with vehicle in several other studies (Seo et al., 1999; Kwak et al., 2000). Measurement of the area of choroidal neovascularization by image analysis confirmed that there was significantly less neovascularization in eyes treated with VEGF-$TRAP_{R1R2}$ compared to those treated with Fc fragment (Fig. 1I). The level of VEGF-$TRAP_{R1R2}$ was measured in plasma obtained from each of the mice at the time of sacrifice. Each of the mice that had been injected with Fc fragment had no detectable VEGF-$TRAP_{R1R2}$ in its plasma, while mice that had been injected with VEGF-$TRAP_{R1R2}$ had plasma levels ranging from 57 to 205 μg/ml. All of the plasma levels of VEGF-$TRAP_{R1R2}$ between 57 and 205 μg/ml were associated with strong inhibition of choroidal neovascularization (Fig. 1J).

Immediately after laser, some mice were given intraocular injection of VEGF-$TRAP_{R1R2}$ or Fc fragment of IgG. Two weeks later, mice were perfused with fluorescein-labeled dextran and choroidal neovascularization was measured. Mice that received intraocular

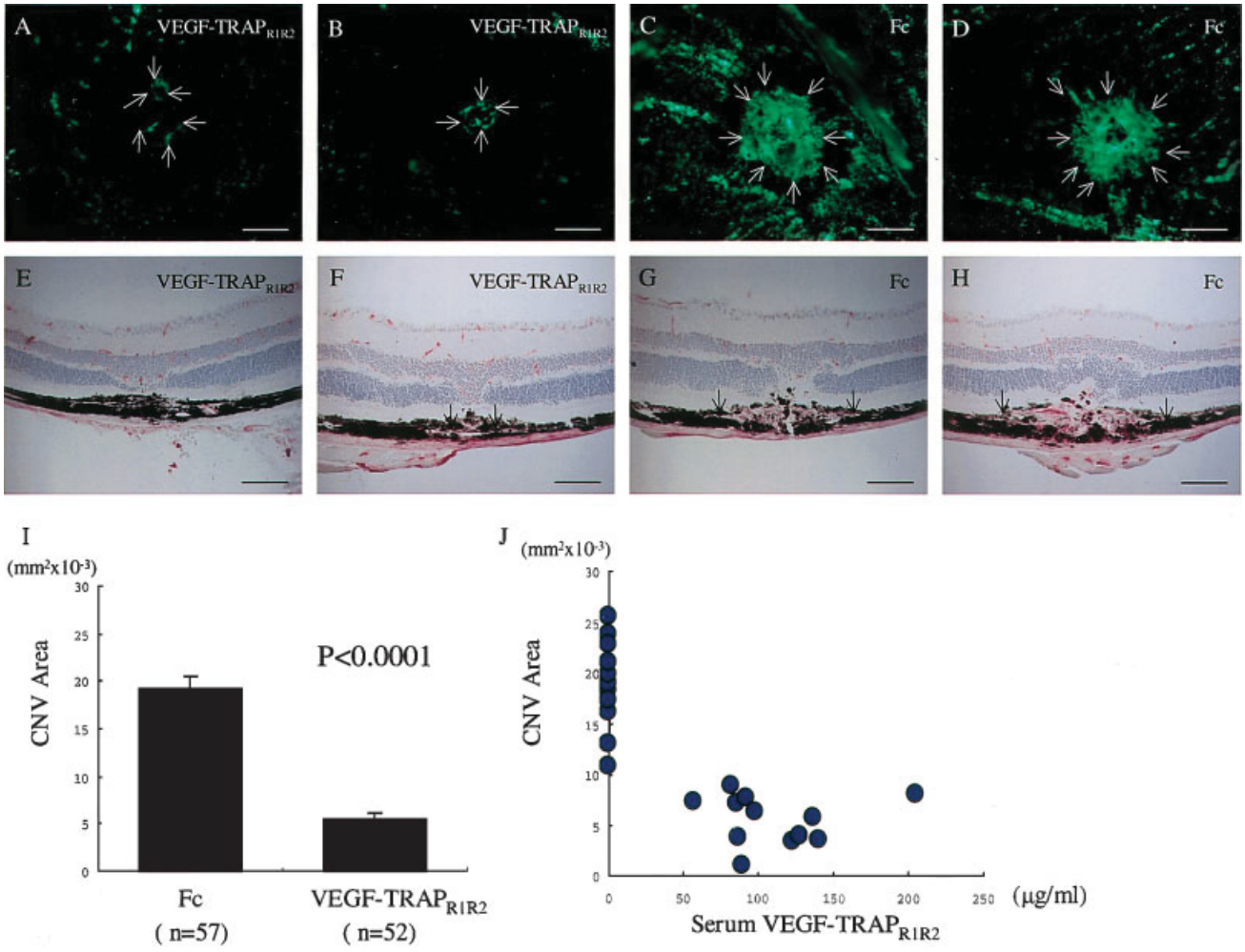

Fig. 1. Subcutaneous VEGF-$TRAP_{R1R2}$ suppresses choroidal neovascularization at sites of rupture of Bruch's membrane. Adult C57BL/6 mice were had rupture of Bruch's membrane by laser photocoagulation in 3 locations in each eye. Prior to laser and on days 2, 5, 8, and 11 after laser, mice received subcutaneous injection of 25 mg/kg of VEGF-$TRAP_{R1R2}$ or Fc fragment of IgG. Parts **A** and **B** show small areas of neovascularization (surrounded by arrows) in retinal whole mounts from two fluorescein dextran-perfused mice treated with VEGF-$TRAP_{R1R2}$. *Griffonia simplicifolia* (GSA) lectin-stained sections in two other mice treated with VEGF-$TRAP_{R1R2}$ show minimal choroidal neovascularization (**E**- none visible and **F**- between arrows). Parts **C** and **D** show large areas of neovascularization (surrounded by arrows) in choroidal flat mounts from two Fc fragment-treated mice and GSA-stained sections from two other mice treated with Fc fragment show prominent areas of neovascularization (**G** and **H**, between arrows). Measurement by image analysis of the area of neovascularization on choroidal flat mounts (**I**) showed an average area that was significantly smaller ($P < 0.0001$ by Student's two-tailed t-test) in VEGF-$TRAP_{R1R2}$-treated mice (20 eyes, 52 rupture sites) compared to Fc-treated mice (20 eyes, 57 rupture sites). Plasma levels of VEGF at the time of sacrifice determined by ELISA plotted against the average area of choroidal neovascularization per mouse showed marked suppression of neovascularization at all plasma levels between 50 and 200 μg/ml (**J**). Bar = 100 μm.

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