A Subretinal Matrigel Rat Choroidal Neovascularization (CNV) Model and Inhibition of CNV and Associated Inflammation and Fibrosis by VEGF Trap

Jingtai Cao,1,2 Lian Zhao,2,3 Ying Li,1 Yang Liu,1 Weibong Xiao,1 Ying Song,3 Lingyu Luo,4 Deqiang Huang,4 George D. Yancopoulos,1 Stanley J. Wiegand,1 and Rong Wen4

PURPOSE. The exudative, or the wet form of age-related macular degeneration (AMD) is characterized by choroidal neovascularization (CNV). A subretinal Matrigel (BD Biosciences, Bedford MA) model of CNV is described here, along with the effects of vascular endothelial growth factor (VEGF) neutralization on the development of CNV and associated inflammation and fibrosis.

METHODS. CNV was induced in adult Sprague-Dawley rats by subretinal injection of Matrigel. CNV growth and associated leukocyte infiltration and collagen deposition were examined. VEGF Trap (Regeneron Pharmaceuticals, Tarrytown, NY), a recombinant protein that comprises portions of the extracellular domains of VEGF receptors 1 and 2 and that binds all isoforms of VEGF-A as well as placental growth factor with high affinity, was administered subcutaneously.

RESULTS. Initiation of CNV was detected 4 days after Matrigel injection and then increased progressively in size. Systemic administration of VEGF Trap beginning on day 2 and 6 completely prevented development of CNV. When CNV was allowed to develop for 10 days before treatment was initiated, VEGF Trap not only prevented its further progression, but also induced substantial regression of existing lesions. In addition, VEGF Trap treatment reduced the total lesion volume and largely prevented the progressive leukocyte infiltration and fibrosis associated with CNV.

CONCLUSIONS. The subretinal Matrigel CNV model provides a convenient tool for the study of the diverse components of complex CNV lesions. The data not only confirm the critical roles of VEGF in the development and maintenance of CNV, but further demonstrate that VEGF and other VEGF receptor 1 ligands promote CNV-associated inflammation and fibrosis.


From 1Regeneron Pharmaceuticals, Inc., Tarrytown, New York; the 2Department of Ophthalmology, University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania; and the 3Bascom Palmer Eye Institute, University of Miami, Miller School of Medicine, Miami, Florida. 4These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Corresponding author: Rong Wen, Bascom Palmer Eye Institute, University of Miami, Miller School of Medicine, 506 McKnight Building, 1638 NW 10th Avenue, Miami, FL 33136; rwen@med.miami.edu.
MATERIALS AND METHODS

Animals and Subretinal Injections

All animal procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult Sprague-Dawley rats (2–3 months old) were purchased from Harlan Laboratories (Indianapolis, IN) or Taconic Farm (Germantown, NY). Subretinal injections of Matrigel (growth factor–reduced synthetic matrix; BD Biosciences) were performed on the temporal side, as described. Briefly, rats were anesthetized with ketamine (40 mg/kg, IP) and xylazine (6 mg/kg, IP). A 33-gauge needle was inserted between the limbus and the equator to reach the subretinal space. A blunt 33-gauge needle attached to a 10-μL microsyringe (Hamilton, Reno, NV) was then introduced into the subretinal space, to inject 1.2 μL of Matrigel, diluted 3:1 with phosphate-buffered saline (PBS; 75% gel).

Visualization of Blood Vessels

Blood vessels were labeled with DiI, (1,1dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Sigma-Aldrich, St. Louis, MO), as described. The animals were euthanatized by CO2 inhalation and perfused with PBS, followed by the DiI solution and 4% paraformaldehyde (in 0.1 M phosphate buffer; pH 7.4). The eye cups were embedded in 5% agarose. Thick (100 μm) serial sections were cut on a soft tissue microtome (Vibratome model VT1000S; Leica Microsystems, Bannockburn, IL) and examined by confocal microscopy. Three-dimensional (3-D) reconstruction was performed using software (AutoVisualize 3-D; AutoQuant Image, Inc. Watervliet, NY). Alternatively, serial frozen sections (50 μm) were cut through the area of the Matrigel deposit and examined by fluorescence microscopy.

Measurement of CNV

CNV area was calculated through the entire Matrigel area (Fig. 1). The CNV area of a section was calculated by multiplying the width (W), the maximum measurement of CNV along the sclera, by the thickness of the section T. The entire CNV area of each eye (Ci) was calculated according to the equation:

\[ C_i = T_i \sum W_i \]

Histologic Evaluation of Lesion Volume, Inflammation, and Fibrosis

To study lesion volume, we fixed the eye cups in 4% paraformaldehyde and embedded them in optimal cutting temperature (OCT) compound (Miles Inc., Elkhart, IN). Serial cryosections of 50 μm were cut through the entire Matrigel area. The area of the lesion in every third section was measured as the area between photoreceptors and the choriocapillaris. The total lesion volume was calculated using the Cavalieri method. Briefly, the lesion area in each section was measured, and the total volume (V) of the CNV lesion in each eye was calculated by multiplying the sum of the areas in all sections (A) by the sum of the stepped thicknesses of the sections (T).

For immunofluorescence staining, cryosections of 10 μm were fixed in acetone at −20°C for 10 minutes, then incubated with Cy3-conjugated monoclonal anti-CD45 antibody (BD Pharmingen, San Diego, CA), Cy3-conjugated monoclonal anti-vimentin antibody (Sigma-Aldrich), Cy3-conjugated monoclonal anti-FAP antibody (Sigma-Aldrich), or Cy3-conjugated anti-α smooth muscle actin (αSMA) antibody (Sigma-Aldrich) for 1 hour at room temperature. Cell nuclei were counterstained with 4',6-diamino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA) and examined by fluorescence microscopy.

RESULTS

Inhibition of CNV Development by VEGF Trap

Angiogenic sprouting was detected as early as 4 days after Matrigel injection (data not shown). Extensive neovascular networks from the choriocapillaris had developed in the Matrigel area by 10 days in all eyes (Fig. 2A). In contrast, subretinal injection of sodium hyaluronate (1.2 μL, 10 mg/mL) resulted in local retinal detachment, but only rarely in the appearance of small CNV, confined to the area immediately adjacent to the break in Bruch’s membrane (Fig. 2B). These results indicate that the deposition of ECM, in conjunction with the disruption...
of Bruch’s membrane, greatly facilitates the growth and spread of CNV.

To determine whether pharmacologic inhibition of VEGF could prevent CNV development in this model, we administered VEGF Trap (12.5 mg/kg, SC) on days 2 and 6 after Matrigel injection. Control animals were injected with an equimolar amount of the control protein, human Fc (hFc, 6.25 mg/kg SC). VEGF Trap is a potent inhibitor of VEGF-A, and the related VEGF receptor 1 (VEGFR1) ligands placental growth factor (PIGF) and VEGF-B. It comprises ligand-binding portions of the extracellular domains of human VEGFR1 and -2, which are expressed in sequence with the Fc domain of human IgG.15,23 At a dose of 12.5 mg/kg, VEGF Trap has been shown to effectively suppress pathologic angiogenesis in several disease models.24,25 Substantial development of CNV was seen in every eye in the control group (CNV area: 211.35 ± 146.00 × 10^3 μm^2, n = 12; Figs. 3A, 3C), but was completely absent in the eyes of VEGF Trap–treated animals (CNV area: 0 ± 0 × 10^3 μm^2, n = 12; P < 0.001, t-test; Figs. 3B, 3C). These results confirm that, as in wet AMD in humans, VEGF plays a vital role in induction and development of CNV in the Matrigel model.

**CNV Regression Induced by VEGF Trap**

To evaluate the effect of VEGF Trap on newly formed CNV, we allowed CNV to develop for 10 days, at which time we collected eyes from one group of animals (10-day [10-D] control) and established the pretreatment baseline. The remaining animals received either VEGF Trap (12.5 mg/kg, SC) or hFc (6.25 mg/kg SC) on days 10, 13, and 16. The eyes were collected on day 20 (Fig. 4A).

CNV was well developed in the 10-D control group (CNV area: 244.50 ± 225.21 × 10^3 μm^2, n = 16) and increased in size by day 20 in the hFc-treated group (CNV area: 1274.27 ± 807.18 × 10^3 μm^2, n = 14; Fig. 4B). In contrast, the CNV area in the group treated with VEGF Trap (37.10 ± 45.87 × 10^3 μm^2, n = 14) was only 15% of that in the 10-D control group (Fig. 4B). A replicate experiment produced similar results (data not shown). Representative confocal images of sections from a 10-D control, a 20-day [20-D] VEGF Trap–treated, and a 20-D hFc-treated control eye are presented in Figures 4C, 4D, and 4E, respectively. It is worth noting that CNV was completely absent in 6 of the 14 eyes in the VEGF Trap–treated group. These results indicate that VEGF Trap not only prevented the substantial growth of CNV from 10 to 20 days, but also induced significant regression of the existing CNV.

**Effects of VEGF Trap on the Total Lesion Volume and Cellularity**

In addition to suppressing CNV, VEGF Trap appeared to substantially reduce the overall volume of the Matrigel lesion. In a separate experiment, we measured the total lesion volume, defined as the area lying between the photoreceptors and the choriocapillaris. The total lesion volume in the 10-D control eyes was 6.7 ± 1.2 × 10^6 μm^3 (n = 10) and 7.5 ± 0.9 × 10^6 μm^3 (n = 8) in the 20-D hFc-treated control eyes. In contrast, the total lesion volume in the VEGF Trap–treated eyes was 2.0 ± 0.2 × 10^6 μm^3 (n = 10), one third of that in the 10-D control group (Fig. 5D). Representative images from 10-D control, 20-D hFc-treated control and 20-D VEGF Trap–treated retinas are shown in Figures 5A, 5B, and 5C, respectively.
The number of cells present in the Matrigel deposit increased markedly between days 10 and 20 in the hFc-treated control group, whereas the reduction in lesion volume in the VEGF Trap-treated group was associated with a near complete inhibition of the progressive increase in cellular density, accompanied by an ongoing clearance of the Matrigel (Fig. 6).

**Inhibition of Progressive Leukocyte Infiltration and Fibrosis by VEGF Trap**

CD45, a pan-leukocyte marker, was used to characterize leukocyte infiltration into the lesion area. Infiltration was evident in the subretinal space and to a lesser degree within the Matrigel deposit in the 10-D control group (Fig. 7A). An increase was seen in the 20-D control group, particularly within the Matrigel lesion (Fig. 7B). This progressive increase in leukocyte infiltration was abrogated by VEGF Trap treatment (Fig. 7C). Semiquantitative analysis confirmed the progression in leukocyte infiltration between 10- and 20-D control eyes, and the suppression of the infiltration by VEGF Trap treatment (Table 1). Moreover, CD45-negative cells, visualized by the nuclear DAPI counterstain seemed to be similarly affected (Fig. 7C).

Fibroblasts and other cells of mesenchymal origin were identified by using vimentin as a marker. In normal eyes (no Matrigel injection), Müller glial cells and fibroblasts in the sclera were well stained, but there was no vimentin staining in the subretinal space (Fig. 8A). In the 10-D group, many vimentin-positive cells were present in and around the Matrigel (Fig. 8B). Vimentin staining was even more prominent in the 20-D control group with a similar staining pattern to that of the 10-D group (Fig. 8C). In contrast, the density of vimentin-positive cells in the VEGF Trap–treated eyes was similar to that in the 10-D control eyes (Fig. 8D). Particularly strong vimentin staining was seen at the border between the retina and the lesion in all groups. This appeared to reflect both the accumulation of vimentin-positive cells at the photoreceptor–Matrigel interface and also increased vimentin staining in the Müller cells. Vimentin staining was strongest in this area in the 20-D control eyes, in which vimentin-positive cells also were dispersed throughout the lesion. Semiquantitative analysis demonstrated that vimentin staining was significantly increased in the 20-D hFc-treated controls when compared with 10-D controls, and VEGF Trap treatment inhibited this increase (Table 1).

**Figure 4.** CNV regression induced by VEGF Trap. (A) Experimental design. CNV was allowed to develop for 10 days in all three groups: untreated 10-D control, 20-D control treated with an inactive protein (hFc), and 20-D group treated with VEGF Trap, at which time the pretreatment baseline CNV area was established by measuring the CNV in the 10-D control group. (B) Extensive CNV had developed in all eyes in the 10-D control group (244.50 ± 225.21 × 10^3 μm^2, n = 16) and was increased further in size in the 20-D control group (1274.26 ± 807.18 × 10^3 μm^2, n = 14). In contrast, the CNV area in the VEGF Trap–treated group (37.10 ± 45.87 × 10^3 μm^2, n = 14) was reduced to approximately 15% of the pretreatment baseline Representative images of the 10-D control, VEGF Trap–treated, and 20-D control groups are presented in (C), (D), and (E), respectively. Arrowheads, CNV. *P < 0.05, **P < 0.01, and ***P < 0.001, respectively (Kruskal-Wallis test and Dunn test). Ch, choroid; M, Matrigel layer; R, retina. Scale bar, 100 μm.

**Figure 5.** VEGF Trap–induced decrease in total lesion volume. Treatment was as described in Figure 4A. Total lesion volume was measured as the area between photoreceptors and the choriocapillaris. Representative images of sections from the 10-D control, 20-D control, and 20-D VEGF Trap–treated groups are shown in (A), (B), and (C), respectively. In addition to reducing the CNV area, the total lesion volume, defined as the entire mass lying between the photoreceptor layer (line) and the choroid, was reduced by VEGF Trap treatment. (D) The total lesion volume was 6.7 ± 1.2 × 10^6 μm^3 (n = 10) in the 10-D control and 7.5 ± 0.9 × 10^6 μm^3 (n = 8) in the 20-D control. In comparison, the total volume in the VEGF Trap–treated group was significantly reduced to 2.0 ± 0.2 × 10^6 μm^3 (n = 10), one third of that in the 10-D control, and 27% of that in the 20-D hFc controls. **P < 0.01 (ANOVA and Tukey test). Ch, choroid; M, Matrigel layer; R, retina. Scale bar, 100 μm.
We next used αSMA to identify smooth muscle cells and myofibroblasts. In the normal retina, αSMA-positive cells were present only in blood vessels at the retinal surface and the inner retina, as well as in blood vessels in the choroid and sclera (Fig. 9A). Ten days after Matrigel injection, αSMA-positive cells were present at the interface between the retina and the Matrigel. These cells were not associated with blood vessels (Fig. 9B). The number of αSMA-stained cells was further increased at 20 days in the hFc-treated controls (Fig. 9C). Thus, within the lesion, the distribution of αSMA-positive cells was similar to that of vimentin-positive cells, particularly at the boundary between the Matrigel deposit and neuronal retina. Treatment with VEGF Trap not only prevented this increase but significantly reduced the extent of αSMA-positive staining, when compared to the 10- and 20-D control groups (Fig. 9D, Table 1). Therefore, in addition to inhibiting the accumulation of mesenchymal cells in and around the Matrigel deposit, VEGF Trap appeared to inhibit the expression of the myofibroblast phenotype by these cells.

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Given the progressive accumulation of a large number of αSMA- and vimentin-positive cells in the lesions, we next evaluated the pattern of collagen deposition in the Matrigel area by using Masson's trichrome stain. With this stain, collagen stains dark blue, whereas the Matrigel itself stains pale blue (Fig. 10A). Collagen staining was very strong in the sclera, but was minimal in and around the Matrigel deposits in the 10-D control eyes. However, focal deposition of collagen was clearly evident in and around the lesions in the 20-D control group (Fig. 10B), especially at the border between the retina and the Matrigel, where vimentin and αSMA-positive cells were most densely and consistently aggregated. In contrast, less collagen staining was visible in animals treated with VEGF Trap than in the 20-D controls (Fig. 10C, Table 1).

Using RPE65 as a marker, we confirmed that subretinal injection of Matrigel induced a rapid translocation of RPE cells from their original position next to Bruch’s membrane, to the opposite side of the Matrigel mass immediately subjacent to the photoreceptors, consistent with earlier findings. This migratory response of the RPE was not inhibited by treatment with VEGF Trap (data not shown).

**DISCUSSION**

We have developed a Matrigel CNV model in rats, to study the mechanisms underlying CNV development and to evaluate...
potential anti-CNV treatments. Matrigel, an extract of extracellular matrix proteins from the murine EHS (Engelbreth-Holm-Swarm) tumor,\textsuperscript{26,27} has been widely used as reconstituted basement membrane in cell culture, as well as in the Matrigel plug assay, to assess angiogenic or anti-angiogenic agents in vivo.\textsuperscript{28} Matrigel also promotes tubulogenic behavior of endothelial cells in vitro.\textsuperscript{29} The subretinal Matrigel model is unique in several ways. First, CNV is initiated by subretinal deposition of ECM. Sub-RPE accumulation of ECM is associated with CNV in wet AMD, and it has been thought to play a role in the induction of neovascularization.\textsuperscript{30} The present work provides further evidence to support a role for ECM in facilitating CNV development. In addition, the CNV lesions were shown to increase progressively in size in this model (Fig. 4). Finally, this model is characterized by a progressive infiltration of leukocytes and myofibroblasts into the developing CNV.

We have reported that subretinal Matrigel deposition induces RPE translocation and CNV in rodents (Wen R, et al. IOVS 2002;43:ARVO E-abstract 1297).\textsuperscript{6} In rabbits, subretinal injection of Matrigel was also reported to be associated with fluorescein leakage,\textsuperscript{7} which was sustained for weeks in some animals, indicating that the blood vessels newly formed by this method are highly permeable. In the present work, we have further characterized the progression and histopathologic characteristics of these lesions in rats. By directly measuring the size of the CNV network, we documented a progressive increase in CNV size for up to at least 20 days after Matrigel injection (Fig. 4). In addition, the entire lesion mass was shown to progress in size (Fig. 5). This increase was associated with a progressive infiltration of nonvascular cells into the developing CNV lesions, notably leukocytes and myofibroblasts, accompanied by deposition of collagen. These features of the subretinal Matrigel model resemble the inflammatory reaction and fibrosis that are well recognized elements of CNV lesions in wet AMD.\textsuperscript{30,31} Thus, the rat Matrigel model not only provides a tool for the study of mechanisms involved in the induction and early progression of CNV, but also to evaluate the effects of potential therapies on diverse aspects of CNV development.

Several animal models of experimental CNV have been developed. The most widely used employs laser photocoagulation to disrupt Bruch’s membrane.\textsuperscript{32} First characterized in primates,\textsuperscript{33} it has been used to induce CNV in other species, including rabbits,\textsuperscript{34} rats,\textsuperscript{35,36} and mice.\textsuperscript{37} Photocoagulation, however, also directly damages the choroid, RPE and overlying retina. In contrast, selective damage to Bruch’s membrane, either mechanical or enzymatic, has met with only limited success in inducing subretinal neovascularization in primates.\textsuperscript{38} Subretinal delivery of adenovirus or adenovirus-associated virus carrying a VEGF transgene has been used successfully to induce subretinal neovascularization in rats.\textsuperscript{39–41} Subretinal injection of FGF2-impregnated gelatin microspheres also has been reported to induce CNV in more detail.
than 80% of injected eyes in rabbits, and VEGF-impregnated gelatin microspheres induces CNV in more than 90% of injected eyes in primates.

Other CNV models have been developed in genetically altered animals. For example, mice with a spontaneous, autosomal semidominant mutation in the Bst locus were reported to exhibit CNV and retinal detachment by 7 months or older, but no basal deposits were found. These animals also exhibit many developmental abnormalities in the retina and eye. Mice lacking monocyte chemoattractant protein-1 (MCP-1; also known as Ccl-2) or its cognate C-C chemokine receptor-2 (Ccr-2) have been reported to develop pathologic features resembling human AMD, including accumulation of lipofuscin in RPE cells, drusen-like deposits, photoreceptor atrophy, and CNV. However, Luhmann et al. recently showed that the inhibition of CNV with VEGF Trap is associated with CNV progression, resulting in a significant reduction in overall lesion volume.

In addition to being a critically important angiogenic factor, VEGF-A is known to promote vascular permeability and inflammation. For example, infusion of exogenous VEGF into the brain can induce vascular leak and influx of inflammatory cells at doses that are insufficient to induce angiogenesis. At higher doses, leukocyte infiltration precedes the initiation of angiogenesis. Similarly, after corneal injury VEGFR1-mediated leukocyte influx precedes and amplifies the subsequent VEGF-dependent neovascularization. The findings in the present work are consistent with a central role for VEGF in mediating the inflammatory reaction associated with CNV formation. However, the inhibitory effects of VEGF Trap on inflammation may not be due solely to its ability to bind VEGF-A isoforms with high affinity, as this receptor-based agent also binds and neutralizes the VEGF1 ligands PlGF and VEGF-B.

In contrast to VEGFR2, which is expressed predominantly on vascular endothelial cells, VEGFR1 is also expressed by a variety of nonendothelial cell types, including subpopulations of smooth muscle cells, leukocytes, and their progenitors. PlGF, in particular, is known not only to synergize with VEGF to promote pathologic angiogenesis, but also to act as a chemoattractant for monocytes and macrophages. Like VEGF, PlGF is present in human CNV membranes, and animal studies have shown that PlGF contributes to the development of experimental CNV.

FIGURE 9. αSMA immunoreactive cells. Treatment was as described in Figure 4A. Retinal sections were stained with anti-αSMA antibodies (red) to identify smooth muscle cells and myofibroblasts. Cell nuclei were counterstained with DAPI (blue). In normal control eyes without Matrigel injection (A), αSMA immunoreactivity was restricted to blood vessels (arrow) in the retina (R), choroid (Ch), and sclera (S) (arrow). Ten days after Matrigel injection (B), many αSMA-positive cells were present in the Matrigel (M), with particularly intense αSMA staining in cells present at the border between the Matrigel and photoreceptors (arrowbeads), an area that was devoid of blood vessels. The staining of αSMA in the 20-D control (C) was similar to that in the 10-D control (B), although more intense at the retina–Matrigel boundary and with more αSMA-positive cells dispersed throughout the Matrigel (arrowbeads). In VEGF Trap–treated animals (D), fewer αSMA-positive cells were found in the Matrigel area (arrowbead) than in the 10-or 20-D control eyes. Ch, choroid; M, Matrigel layer; R, retina; S, sclera. Scale bar, 50 μm.
In summary, we have developed a CNV model in the rat by subretinal deposition of Matrigel, which exhibits many features of human wet AMD. Using this model, we have shown that VEGF Trap, a potent receptor-based inhibitor of VEGF-A and PlGF, not only arrests the growth of experimental CNV, but also the associated inflammatory and fibrotic responses and can induce regression of recently established lesions. VEGF Trap, a potent receptor-based inhibitor of VEGF-A and PlGF, not only arrests the growth of experimental CNV, but also the associated inflammatory and fibrotic responses and can induce regression of recently established lesions.

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**Figure 10.** Collagen deposition. Treatment was as described in Figure 4A. Collagen was visualized by Masson’s trichrome stain: collagen components are stained dark blue, and the Matrigel is pale blue. Minimal collagen deposition (arrows) was seen in the 10-D control (A), whereas collagen deposition (arrows) was clearly evident in the 20-D control (B), most notably at the boundaries between the Matrigel deposit and the retina and to a somewhat lesser extent at the boundary with the choroid. In VEGF Trap–treated retinas (C), there was minimal collagen deposition (arrow), similar to that in 10-D control eyes. S, sclera; Ch, choroid; M, Matrigel layer; R, retina. Scale bar, 50 μm.


