Pathophysiological Role of VEGF on Retinal Edema and Nonperfused Areas in Mouse Eyes With Retinal Vein Occlusion

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Purpose. To determine the relationship between retinal morphologic changes and molecules involved in the changes after anti-VEGF treatment in the retina of retinal vein occlusion (RVO) murine model.

Methods. The studies were performed on murine RVO model created by laser irradiation of retinal veins. The site of VEGF expression was determined by immunostaining, and the retinal thickness was measured in the images obtained by optical coherence tomography. The levels of VEGF-related and inflammatory factors after an intravitreal injection of anti-VEGF antibody immediately or 7 days after laser irradiation were determined by Western blotting.

Results. The level of VEGF increased in all retinal layers 1 day after laser irradiation, and expression was higher in the partially perfused areas than in the completely nonperfused areas. In eyes with high expression level of VEGF, early administration of anti-VEGF antibody reduced the retinal thickness, and expressions of VEGF and inflammatory factors returned to normal levels. However, the levels of phosphorylated protein kinase B (AKT), extracellular signal-regulated kinase 1 and 2 (ERK1/2), and endothelial nitric oxide synthase (eNOS) were increased by early administration of anti-VEGF antibody. On the other hand, in eyes with low concentration of VEGF, late injection of anti-VEGF antibody induced retinal thinning and the concentrations of phosphorylated AKT, ERK1/2, and eNOS were lower than that in normal group. Furthermore, anti-VEGF antibody lessened the reduction of aquaporin-4.

Conclusions. These findings indicate that the effect of anti-VEGF antibody is most likely dependent on its effect on the intraocular VEGF levels.

Keywords: retinal vein occlusion, anti-VEGF antibody, blood flow, macular edema

The worldwide prevalence of retinal vein occlusion (RVO) is about 5.2 per 1000 individuals, and the total number of individuals with RVO is about 16 million. A RVO can cause significant ocular morbidity including macular edema which can then reduce the visual acuity. The degree of visual impairment is strongly correlated with the degree macular edema and the sizes of the nonperfused areas.

Once the retina becomes ischemic by the reduction of blood supply caused by the RVO, the level of expression of VEGF is increased in the intraretinal tissues. It has been reported that VEGF is highly involved in the development of the edema and the sizes of the nonperfused areas in eyes with RVO. Although VEGF plays an important role in maintaining the physiologic condition of the retina under the normal conditions, excess VEGF can cause pathologic alterations including a hyperpermeability of the retinal capillaries and migration and proliferation of the retinal endothelial cells. The upregulation of VEGF is associated with the pathologic signs and symptoms of ischemic retina in RVO patients. The primary cause of the edema in eyes with RVO is the breakdown of the blood-retinal barrier (BRB) that is formed by the vascular endothelial cells and the RPE cells by the excess VEGF. Moreover, the nonperfused areas in eyes with RVO increase the hydrostatic pressure in the blood vessels that then disrupts the blood flow. The damaged capillaries can lead to a permanence of the nonperfused areas by the action of vasoproliferative agents such as VEGF that are released under the retinal hypoxic conditions. Thus, it is important to investigate the site of VEGF expression to determine the mechanisms causing the retinal edema and ischemic areas in eyes with a RVO.

Anti-VEGF antibody is now the first choice treatment for eyes with a RVO. The intravitreal administration of anti-VEGF antibody has been shown to ameliorate the retinal edema and reduce the size of the nonperfused areas in eyes with a RVO. However, some patients still have poor vision because of repeated recurrences of the macular edema and the development of nonperfused areas despite the administration of the anti-VEGF antibody. The use of anti-VEGF antibody on RVO patients has led to contradictory findings on the signs and the symptoms of RVO. Retinal edema and nonperfused areas are formed 1 day after laser irradiation in the murine RVO model. In addition, the retinal thickness is decreased and nonperfused areas are formed 7 days after the laser irradiation. We have confirmed these findings in mice that have had an intravitreal injection of mouse anti-VEGF antibody immediately or 7 days after the laser irradiation. The size of the nonperfused areas was smaller if the anti-VEGF antibody was injected immediately after the laser irradiation in RVO murine...
model. On the other hand, the size of the nonperfused areas was larger if the anti-VEGF antibody was injected 7 days after the laser irradiation. The effects of anti-VEGF antibody against the edema and nonperfused areas are still not well understood because of the differences in the findings associated with the time of the anti-VEGF antibody injection.

The aim of this study was to determine the relationship between the pathologic morphologic changes and the expression of the hyperpermeability factors in mice with RVO. In addition, we investigated to determine the changes in the VEGF-related molecules and inflammatory factors after an early or late administration of anti-VEGF antibody in the murine RVO model.

**Materials and Methods**

**Study Approval**

All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the experimental protocols were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

**Animals**

We purchased 8-week-old male ddY mice from Japan SLC (Hamamatsu, Japan) and caged in our animal facilities which was maintained at 25°C ± 3°C under 12-hour light and 12-hour dark schedule. The mice had ad libitum access to food and water.

**Retinal Vein Occlusion (RVO) Mouse Model**

The RVO mouse model was created as described in detail. Briefly, the mice were anesthetized with a mixture of ketamine (120 mg/kg; Daiichi-Sankyo, Tokyo, Japan) and xylazine (6 mg/kg; Bayer, Health Care Osaka, Japan). The pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen Pharmaceutical Co., Ltd, Osaka, Japan), and hydroxyethyl cellulose (Senju Pharmaceutical Co. Ltd., Osaka, Japan) was applied to the cornea to prevent desiccation. Three retinal veins were photocoagulated by a 532-nm laser light applied at 50 mW, 5 seconds, and 50 μm (Phoenix Research Laboratories, Inc., Pleasanton, CA, USA). The right eye of each animal was irradiated after the injection of rose bengal (8 mg/mL; Wako, Osaka, Japan) into a tail vein. Then, 10 to 15 laser spots were applied to three retinal veins of each mouse at three discs diameters from the optic nerve head.

**Drug Administration**

Mouse anti-VEGF antibody (200 ng/mL; R&D Systems, Minneapolis, MN, USA) was injected (2 μL) into the vitreous body of the right eyes immediately or 7 days after the laser irradiation as reported. A sterile 34-gauge needle (Terumo, Tokyo, Japan) was attached to a Hamilton glass syringe (701 N; Hamilton Co., Reno, NV, USA) and filled with the anti-VEGF antibody. For controls, mice were injected intravitreally with 2 μL of 0.01 M phosphate buffered saline into the right eyes. After the intravitreal injection, 0.5% levofloxacin ophthalmic solution (Santen Pharmaceuticals Co., Ltd.) was applied topically to the treated eyes.

**Western Blot Analysis**

Mice were euthanized by cervical dislocation, and the eyes were rapidly removed. The retinas were carefully separated from the retinal pigment epithelium, and the isolated retina was quickly frozen in dry ice. The tissue was homogenized in cell lysis buffer using a homogenizer (Physcotron; Microtec Co., Ltd., Chiba, Japan). The retinal lysate was centrifugated at 12,000 g for 20 minutes, and the protein concentration of the supernatant was measured with bovine serum albumin with a biinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA). A mixture of equal parts protein and sample buffer with 10% 2-mercaptoethanol was separated on a 5% to 20% SDS-PAGE gradient gel, and the proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Billerica, MA, USA). The transferred membranes were blocked for 30 minutes at room temperature with blocking solution (Blocking One-P; Nakalai Tesque, Inc., Kyoto, Japan), then incubated overnight at 4°C with the different primary antibodies. The following primary antibodies were used for immunoblotting: VEGF (rabbit-derived, 1:100; Merck Millipore, Billerica MA, USA), glial fibrillary acidic protein (GFAP; mouse-derived, 1:500; Cell Signaling, Danvers MA, USA), hypoxia-inducible factor-1 (HIF-1α; rabbit-derived, 1:1000; Abcam, Cambridge, MA, USA), intercellular adhesion molecule-1 (ICAM-1; mouse-derived, 1:200; Santa CruzBio-technology, Inc., Dallas, TX, USA), rabbit anti-phosphorylated-protein kinase B (p-AKT; 1:1000; Cell Signaling), rabbit anti-total-AKT (1:1000; Cell Signaling), rabbit anti-phosphorylated-extracellular signal-regulated kinase and 1 and 2 (p-ERK1/2; 1:1000; Cell Signaling), rabbit anti-total-ERK1/2 (1:1000; Cell Signaling), rabbit anti-phosphorylated-endothelial nitric oxide synthase (p-eNOS; 1:1000; Cell Signaling), rabbit anti-total-eNOS (1:1000; Cell Signaling), and mouse anti-β-actin antibody (1:2000; Sigma-Aldrich Corp., Tokyo, Japan). The secondary antibodies were goat anti-rabbit horseradish peroxidase (HRP-) conjugated or goat anti-mouse HRP-conjugated (Pierce Biotechnology, Inc, Waltham, MA, USA). The immunoreactive bands were made visible by a luminol derivative (ImmunoStar technology, Inc, Waltham, MA, USA). The immunoreactive bands were made visible by a luminol derivative (ImmunoStar technology, Inc, Waltham, MA, USA). The immunoreactive bands were made visible by a luminol derivative (ImmunoStar technology, Inc, Waltham, MA, USA). The immunoreactive bands were made visible by a luminol derivative (ImmunoStar technology, Inc, Waltham, MA, USA).
antibodies were: VEGF (1:50; Merck Millipore), mouse anti-GFAP (1:500; Santa Cruz Biotechnology, Inc.), and rabbit anti-aquaporin-4 antibody (AQP4; 1:1000; Sigma-Aldrich Corp.). The secondary antibodies were goat anti-rabbit conjugated with AlexaFluor 488 (1:1000; Thermo Fisher Scientific) and goat anti-mouse conjugated with AlexaFluor 546 (1:1000; Thermo Fisher Scientific). The sections were photographed with a fluorescence microscope (BXZ710; Keyence, Osaka, Japan) or a confocal microscope (FV10i; Olympus Corp., Tokyo, Japan). For quantitative data, images were taken 500 μm from the optic nerve head.

Staining of Whole-Mount Preparations. The mice were injected into the tail vein with 0.5 mL of 20 mg/mL fluorescein conjugated dextran (Sigma-Aldrich Corp.) dissolved in PBS. The mice were killed, and the eyes were enucleated and fixed for 1 hour in 4% PFA. The anterior segment was removed, and the eyecup was incubated for another 50 minutes in 4% PFA. The retina was then isolated and flat-mounted on glass slides. The slides were incubated in blocking solution consisting of 10% normal goat serum in PBS containing 0.3% Triton X-100 for 1 hour at room temperature. The retinas were incubated for 24 hours with primary antibodies (VEGF 1:50; Merck Millipore) at 4°C. Then the retinas were stained with isoeinconjugated dextran from Griffonia simplicifolia conjugated to AlexaFluor 594 (20 μg/mL; Thermo Fisher Scientific) overnight at 4°C. Then, the retinas were incubated for 1 hour with secondary antibodies (AlexaFluor 633, 1:200; Thermo Fisher Scientific) at room temperature. The sections were photographed with a confocal microscope (Olympus Corp.).

Optical Coherence Tomography

The retinal thickness was measured in the optical coherence tomography (OCT; 830 nm) images obtained by a fundus camera (Micron IV; Phoenix Research Laboratories, Inc.) and an OCT scan head equipped with a mouse objective lens (Phoenix Research Laboratories, Inc.) as reported in detail. Briefly, the scanned region on the mouse retina was 1.8 mm in the x and y directions, and the linear OCT scans consisted of a series of 1024 single point A-scans. The pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen Pharmaceutical), and hydroxyethyl cellulose (Senju Pharmaceutical) was used to prevent the cornea from dehydrating. The images were taken at 20 positions of the right eyes by commercial software (StreamPix 6 and Micron OCT; Phoenix Research Labs). The retinal thickness was measured at 20 positions by the software of Insight, and the average of all positions was used for the overall retinal thickness.

Blood Flow Measurements by Laser Speckle Flowgraphy

The blood flow on the optic nerve head was measured by laser speckle flowgraphy (LSFG; Softcore Co., Ltd., Fukuoka, Japan) as reported in detail. The blood flow was represented by the mean blur rate (MBR), which is an index of the blood flow velocity. The images used to determine the MBR were taken at a rate of 30 frames/sec over a time period of about 4 seconds. The measured fundus area was 3.8 × 3 mm (width × height) with a tissue penetration of 0.5 to 1 mm. The vessel and tissue areas on the optic nerve head area were automatically detected by the commercial software (LSFG Analyzer version 3.1.14.0; Softcore Co., Ltd., Tokyo, Japan). The MBR of the three areas of the optic nerve head were calculated by the software; the MBR of the total area (MA) was measured as the average of MBR over the entire optic nerve head, the MBR of the vessel region (MV) was calculated as the average of the vessel region, and the MBR of the tissue region (MT) was measured as the average MBR of the total optic nerve head area minus the vessel region.

Statistical Analyses

The data are presented as the means ± SEMs. The significance of the differences was determined by the Student’s t-tests and 1-way ANOVA followed by the Dunnett’s test. The SPSS Statistics (IBM Corp., Armonk, NY, USA) software was used for the statistical analysis. Values of P < 0.05 were taken to be statistically significant.

Results

Increased VEGF expression in Retinas of Mouse Model of RVO

To determine the mechanisms involved in the retinal edema in eyes with RVO, Western blotting was used to measure the expression levels of VEGF-related factors. The results of previous studies showed that the expressions of VEGF and phosphorylated VEGFR2 were increased in the endothelial cells without an increase in the phosphorylated VEGFR1 in an ischemia-induced retinal neovascularization mouse model. In our RVO mouse model, the expression level of VEGF was significantly increased 1 day after the laser irradiation (Fig. 1A), and the expression level of phosphorylated VEGFR2 was significantly increased on days 0.5 to 3 after the laser application. On the other hand, the expression level of VEGFR2 was significantly decreased from days 0.5 to 7 after the laser irradiation (Supplementary Fig. S1A).

In earlier reports, the level of phosphorylated protein kinase B (Akt), extracellular signal-regulated kinase 1 and 2 (ERK1/2), and endothelial nitric oxide synthase (eNOS) signaling pathways were upregulated under ischemic conditions. In our model, the level of phosphorylated Akt was increased on days 0.5 to 7 after the laser application (Fig. 1B). The expressions of the phosphorylated ERK1/2 and eNOS were also significantly increased on days 0.5 and 1 after the laser irradiation (Figs. 1C, 1D).

Level of Expression of VEGF Is Decreased by Anti-VEGF Antibody in RVO Mouse Model

The intravitreal injection of anti-VEGF antibody was associated with changes in the pathologic conditions in the retina under hypoxic and inflammatory conditions. Hypoxia-inducible factor-1α (HIF-1α) plays a role in the upregulation of VEGF in ischemic retina. In addition, the expression level of intercellular adhesion molecule-1 (ICAM-1), an adhesion molecule on endothelial cells is increased by the upregulation of VEGF during inflammation.

Western blotting was used to examine the expression levels of VEGF-related factors and inflammatory factors after either an early or a late injection of anti-VEGF antibody after the laser irradiation. Early injection of anti-VEGF antibody significantly reduced the expression levels of VEGF HIF1α, ICAM-1, and phosphorylated VEGFR2 (Figs. 2C–E, Supplementary Fig. S1B). In addition, the levels of phosphorylated Akt, ERK1/2 and eNOS were increased by an immediate intravitreal injection of anti-VEGF antibody after the laser irradiation (Figs. 2F–H). On the other hand, the levels of expressions of VEGF phosphorylated VEGFR2 and the levels of phosphorylated Akt, ERK1/2, and eNOS were lower than that of the normal group by the late injection of anti-VEGF antibody (Figs. 2K, 2N–P and Supplementary Fig. S1C). Late injection of anti-VEGF antibody did not change the level of HIF1α and ICAM-1 (Figs. 2L, 2M).
Increase of VEGF in All Retinal Layers in RVO Mouse Model

The site of the VEGF expression was determined by immunostaining the retinas of RVO mouse model. The fluorescent intensity of VEGF (green) was increased in all layers on days 1 and 3 after the laser irradiation, and it was not significantly different from that of the normal group after day 7 (Figs. 3A, 3B). The expression level of VEGF was also high in the inner nuclear layer (INL) on days 1 and 3 after the laser irradiation, and retinal edema was present in the INL which is the site of the retinal vessels (Fig. 3C).

To determine which cells were expressing VEGF, we investigated the expression level of GFAP, a marker of astrocytes and activated Müller glial cells, by Western blotting. Earlier studies showed that under retinal ischemic conditions, the astrocytes were activated by the decrease of blood flow, damaged blood vessels, and the production of VEGF-related factors. In our RVO model, the level of expression of GFAP was significantly increased on days 0.5 and 1 after the laser irradiation (Fig. 3D). An early injection of anti-VEGF antibody significantly reduced the level of expression of GFAP (Fig. 3E). We also determined the site of activated astrocytes by immunostaining the retinas of RVO mice. Double staining for VEGF and GFAP showed that the astrocytes were activated in the retinas of the vehicle-treated group (Fig. 3F). VEGF was colocalized with GFAP in the RVO mice. On the other hand, the injection of anti-VEGF antibody reduced the number of

FIGURE 1. Expression levels of VEGF signaling in a mouse RVO model. (A) Level of expression of VEGF in the retina at 0.5, 1, 3, and 7 days after the laser irradiation that occluded three retinal veins and in untreated mice. The VEGF level is significantly increased on day 1 after the laser irradiation. (B) Level of expression of phosphorylated protein kinase B (AKT) in the retina at 0.5, 1, 3, and 7 days after the laser irradiation and in untreated mice. The expression level of phosphorylated AKT is increased on days 0.5 to 7 after the occlusions. (C, D) Level of expression of phosphorylated extracellular signal-regulated kinase 1 and 2 (ERK1/2) (C), and phosphorylated endothelial nitric oxide synthase (eNOS) (D) in the retina of the retinal vein occlusion (RVO) murine model. The expression levels of phosphorylated ERK1/2 and eNOS are significantly increased on day 0.5 and 1 after the laser occlusions. Data are the means ± SEMs (n = 4 or 5). *P < 0.05, **P < 0.01 versus normal (Dunnett’s test).
FIGURE 2. Effects of anti-VEGF antibody on VEGF signaling in mouse RVO model. (A, I) RVO mice were injected intravitreally with anti-VEGF antibody immediately (A) or 7 days (I) after the laser irradiation. The retinas were examined 1 day after the anti-VEGF antibody. (B, J) Representative Western blots showing the expression of VEGF, hypoxia-inducible factor-1 (HIF-1α), intercellular adhesion molecule-1 (ICAM-1), and the VEGF-related factors after the intravitreal injection of anti-VEGF antibody (C–H, K–P). Quantitative analysis of VEGF, HIF-1α, and ICAM-1 with normalization to β-actin (C–E, K–M), phosphorylated Akt (F, N), ERK1/2 (G, O) and eNOS (H, P) with normalization to total Akt, ERK1/2, or eNOS.
activated astrocytes in the retinas of the RVO mouse model. In addition, we double immunostained for VEGF glutamate synthetase (Gs), a marker of Müller cells, Pax6, a marker of amacrine cells, isocitrate B4 (IB4), a marker of vascular endothelial cells, and neuronal nuclei (NeuN), a marker of ganglion cells. VEGF was co-localized with IB4 in the RVO mice (Supplementary Fig. S2D). However, VEGF was not colocalized with Gs, Pax6, and NeuN (Supplementary Figs. S2A–C). These results indicated that the activated astrocytes and vascular endothelial cells were associated with the upregulation of VEGF.

**Increased VEGF Expression at Partially Perfused Areas in RVO Mouse Model and its Reduction by an Early Anti-VEGF Antibody Injection**

We investigated the expression of VEGF in the nonperfused areas by immunostaining retinal flat-mounts to investigate the site of VEGF expression causing the retinal edema and ischemic areas in eyes with a RVO. The retinas were costained with IB4 (red), and FITC-dextran (green), a marker of perfused areas (Fig. 4). The expression of VEGF (blue) in the partially perfused areas was higher than in the completely nonperfused areas, and the increase was suppressed by the injection of anti-VEGF antibody in the early phase (Fig. 4).

**Decrease of Retinal Thickness and Blood Flow After Intravitreal Injection of Anti-VEGF Antibody at Late Phase in RVO Mouse Model**

It has been reported that the time of the injection of anti-VEGF antibody is important for its ability to reduce the size of the nonperfused areas in the RVO mouse model. However, it is still poorly understood how the time of the anti-VEGF antibody injection affects the retinal edema. To examine this, we measured the changes in the retinal thickness in the OCT images and the blood flow by laser speckle flowgraphy in eyes after an early or late injection of anti-VEGF antibody. The results showed that an early injection of the anti-VEGF antibody normalized the retinal pathologic thickening (Fig. 5A) and increased the retinal blood flow in all regions of the optic nerve head of the RVO mouse (Fig. 5B). On the other hand, a late injection of the anti-VEGF antibody increased the degree of reduction of the retinal thickness (Fig. 5C), and the reduction of the retinal blood flow (Fig. 5D).

**Expression Level of AQP4 Is Significantly Increased by Early-Phase Injection of Anti-VEGF Antibody**

AQP4 is a water channel protein and is known to be expressed on retinal Müller cells. It has been reported that the absorption of fluid from the inner retinal tissues is through the AQP4 channels, and retinal ischemia downregulates the activity of AQP4. This then impairs the fluid clearance leading to retinal edema. We have confirmed that the retinal edema was due to the upregulation of VEGF and the downregulation of AQP4 in the mouse RVO model. However, the relationship between the sites of these expressions and the hyperpermeability was not determined. Thus, we investigated the degree of expression of AQP4 by Western blotting and the site of the expression by immunostaining. Our results showed that the expression level of AQP4 was significantly decreased on days 0.5 and 1 after the laser irradiation, however, it was increased on day 7 after the laser irradiation (Fig. 6A). An early injection of the anti-VEGF antibody increased the expression level of AQP4 compared with that of the vehicle-treated group (Fig. 6B). We also confirmed the location of AQP4 by immunostaining. The injection of anti-VEGF antibody immediately after the laser irradiation reduced the degree of downregulation of AQP4 in all retinal layers (Fig. 6C).

**DISCUSSION**

The results showed that the levels of expression of the VEGF-related factors were increased (Fig. 1), and VEGF was colocalized with GFAP and IB4 in the RVO mice (Fig. 3, Supplementary Fig. S2). The level of expression of the VEGF-related factors was higher in the partially perfused areas than in the completely nonperfused areas (Fig. 4). The early injection of anti-VEGF antibody reduced the number of activated astrocytes and the degree of downregulation of AQP4 (Figs. 3, 6). Moreover, an early injection of anti-VEGF antibody after the laser treatment significantly reduced the retinal thickness, the degree of retinal hypoperfusion, and the expression of VEGF, HIF-1α and ICAM-1. However, the levels of phosphorylated AKT, ERK1/2, and eNOS were increased by the early administration of anti-VEGF antibody. In eyes with low concentrations of VEGF, a late injection of anti-VEGF antibody induced retinal thinning and the level of phosphorylated AKT, ERK1/2, eNOS was lower than that in the normal group (Figs. 2, 5).

VEGF is synthesized in the endothelial, amacrine, ganglion, and Müller glial cells under ischemic conditions. In addition, the astrocytes are activated by many factors including the VEGF-related cytokines due to the disruption of the BRB. We found that the expression of VEGF was increased in all retinal layers and in the inner nuclear layer, and was colocalized with GFAP and IB4 (Fig. 3, Supplementary Fig. S2). In addition, our findings indicate that the injection of anti-VEGF antibody in the early phase reduced the degree of activation of GFAP (Fig. 3). The astrocytes play a role in the regulation of blood flow and the maintenance of the vascular structures. Under retinal ischemic conditions, the astrocytes are activated by the decrease of blood flow and damaged blood vessels, and the production of VEGF-related factors. Moreover, VEGF is synthesized in endothelial cells and is a potent inducer of proliferation and migration of endothelial cells. Therefore, the results suggest that the edema is probably associated with the activation of the astrocytes and endothelial cells.

It is important to determine the mechanisms causing the development of the nonperfused areas which leads to a reduction of the visual acuity in eyes with RVO. There are some earlier findings that suggested the enlargement of the nonperfused areas was related to the accumulation of VEGF near
Figure 3. Retinal sites of VEGF expression in mouse RVO model. (A) Representative photomicrographs showing VEGF expression in the retinas at 0.5, 1, 3, and 7 days after the laser irradiation in a mouse RVO model and in untreated mice. The fluorescent intensity of VEGF (green) is increased in all layers (B) and in the INL (C) 1 day after the laser irradiation in RVO mice. The nuclei are stained blue by Hoechst33342. Data are the means ± SEMs. (n = 4 or 5) "P < 0.05, ""P < 0.01 versus normal (Dunnett’s test). INL, inner nuclear layer; ONL, outer nuclear layer. (D) Expression level of glial fibrillary acidic protein (GFAP) in the retina at 0.5 and 1 day after the laser irradiation that occluded three retinal veins and in untreated mice.
The level of expression of GFAP is significantly increased 0.5 and 1 day after the laser irradiation. Data are the means ± SEMs. (n = 6) **P < 0.01 versus normal. (Dunnett’s test) (E) The injection of anti-VEGF antibody immediately after the laser irradiation significantly decreases the GFAP level compared with the vehicle-treated group. Data are the means ± SEMs (n = 5). **P < 0.01 versus normal, *P < 0.01 versus vehicle (Student’s t-test). (F) Representative photographs showing double immunostaining for VEGF and GFAP in the retinas of normal, vehicle and anti-VEGF antibody-treated groups (magnification ×60). The nuclei are stained blue by Hoechst33342, and GFAP (red) is a marker of activated astrocytes. The astrocytes are activated in the retina of the vehicle-treated group, VEGF (green) is colocalized with GFAP in the vehicle-treated group. On the other hand, the injection of anti-VEGF antibody reduces the number of activated astrocytes. GCL, ganglion cell layer; IPL, inner plexiform layer; OPL, outer plexiform layer. Scale bar: 50 μm.

the BRB disturbances. It has been reported that the accumulation of VEGF was associated with the formation of the nonperfused areas. Clinical studies have shown that differences in the loss of superficial and deep capillary plexuses can affect the development of the pathologic signs of RVO. This suggests that the production of VEGF in the nonperfused areas is dependent on the degree of capillary loss.

It has been reported that the level of VEGF around the blood vessels in the peri-ischemic area is increased in the middle cerebral artery occlusion (MCAO) model. We found that the level of VEGF was increased especially in the partially nonperfused areas in the mouse RVO model as well as in the MCAO model, and the level of expression of VEGF was reduced by anti-VEGF antibody (Fig. 4). The findings in an earlier study suggested that the VEGF protein was produced by microglial cells and macrophages that bind to their receptors on neighboring vascular endothelial cells during tissue hypoxia. However, the administration of anti-VEGF does not inhibit the production of VEGF by the retinal vessels and endothelial cells completely. Under normal conditions, VEGF is synthesized by the endothelial cells, therefore VEGF exists in the endothelial cells of the retinal vessels. So, the endothelial cells were immunostained with VEGF in normal eyes and also in the anti-VEGF antibody-treated group. Taken together, these findings indicate that it is important to know that the cause of the development of nonperfused areas is strongly associated with an increase in the levels of VEGF in the nonperfused and partially perfused areas in eye with a RVO.

AQP4 is concentrated in the end-feet of astrocytes that makes contact with the micropalial endothelial cells forming the BRB. The level of AQP4 is decreased in a variety of the pathologic conditions generally by increasing the number of astrocytes and also during glia scar formation.

We have reported that the formation of the edema in RVO is associated with an increase in the expression level of VEGF and AQP4. The level of AQP4 was reduced in the retina of RVO mice, and this decrease was suppressed by the administration of anti-VEGF antibody in early phase (Fig. 6). It has been reported that the level of expression of AQP4 on the astrocytes was decreased, and the level of expression of VEGF was increased by the disruption of water channel balance in a cerebral ischemic model. The development of edema in diabetic retinopathy is associated with a decrease in the expression of AQP4 by an increase of VEGF and NO, and the water transport in the retinal neuronal cells is not stabilized. These findings indicate that AQP4 might regulate the formation and recurrence of edema through VEGF signaling in the mouse RVO model.

The hyperpermeability caused by an elevated VEGF is associated with a breakdown of cardiovascular homeostasis and vessel integrity. These changes act through the ERK1/2 signaling pathway and the NO-mediated synthesis against the ischemic conditions. In earlier reports, the phosphorylated AKT, ERK1/2, and eNOS signaling pathways were upregulated under ischemic conditions. AKT is one of the signaling molecules involved in endothelial cell survival, and it is strongly activated by a variety of growth factors and cytokines except VEGF. In addition, VEGF R2 plays an important role as the endothelial cell receptors for VEGF that are expressed on retinal neurons and endothelial cells. The ischemia-reperfusion injury induces the expression of tight junctions and BRB permeability though the upregulation of phosphorylated VEGF R2. Our results confirmed the increase in the expressions of these VEGF-related factors in the mouse RVO model (Fig. 1). An early administration of anti-VEGF antibody significantly reduced the retinal thickness, the degree of the downregulation of the blood flow, and the level of expression of VEGF, HIF-1α, and ICAM-1. However, the expressions of phosphorylated AKT, ERK1/2, and eNOS were increased by the administration of anti-VEGF antibody in the early phase (Figs. 2, 5). Our findings in the mouse model of RVO indicated that the blood flow on the optic disc was decreased by the laser-induced RVO, and the edema was due to the upregulation of VEGF. It has been reported that the expression of Vegfa and the mRNA of inflammatory genes in sham-operated mice were not altered compared to normal group. These findings indicate that the up-regulation of Vegfa and the mRNA of the inflammatory genes were caused by the occlusion of the retinal vessels. The intravitreal injection of anti-VEGF antibody is associated with the changes of pathologic conditions caused by the reduction of VEGF and inflammatory factors as hypoxia and inflammatory diseases. On the other hand, the phosphorylated AKT, ERK1/2, and eNOS signaling pathways can play a role in the neuroprotection of the retina under ischemic conditions. Therefore, the retinal edema and decrease in the blood flow after the early injection of anti-VEGF antibody was ameliorated by the changes in the expression of VEGF-related and inflammation-related factors that are caused by endothelial cell death in cases of an upregulation of VEGF in the eyes with RVO. On the other hand, a late administration of anti-VEGF antibody significantly reduced the retinal thickness, blood flow, and VEGF-related factors (Figs. 2, 5). A downregulation of VEGF leads to vascular disturbances and the regression of blood vessels because of the downregulation of the VEGF signaling pathways in the endothelial cells. Although VEGF is important for maintaining the homeostasis of the retinal vasculature, the overexpression of the VEGF changes the normal state to a pathologic state including induction of retinal vascular hyperpermeability.

Based on these findings, we suggest that the vasodilation and the decrease in blood flow may be associated with the decrease of VEGF-related factors after the injection of anti-VEGF antibody in cases of a downregulation of VEGF in the RVO model.

We believe that it is not sufficient to determine the relationship between the RVO and VEGF by the appearance in the fluorescein angiograms, and it is important to determine the expression levels of VEGF before and after the injection of anti-VEGF antibody.

We conclude that the effect of anti-VEGF therapy for the retinal edema was due to its effect on the retinal VEGF level. In addition, our results indicate that the early detection of RVO and intervention by anti-VEGF therapy would be advantageous.
#### FIGURE 4. Relationship between VEGF and nonperfused areas in mouse RVO model. Representative photomicrographs of the nonperfused areas and partially perfused areas in flat-mounted retinas from untreated, vehicle-treated, and anti-VEGF antibody-treated groups. The vascular endothelial cells are stained red by IsolectinB4 (IB4). The areas stained by FITC-dextran marker (green) indicate the perfused areas. The expression of VEGF in the partially perfused areas is higher than the completely nonperfused area on day 1 after the injection of anti-VEGF antibody immediately after the laser irradiation. The increase is reduced by the anti-VEGF antibody. Scale bar: 50 μm.

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<th>Condition</th>
<th>Laser Irradiation</th>
<th>Anti-VEGF Antibody</th>
<th>IB4</th>
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**FIGURE 5.** Effects of anti-VEGF antibody on retinal thickness and blood flow in mouse RVO model. (A, B) The retinal thickness was measured in the optical coherence tomographic (OCT) images, and the blood flow was determined by laser speckle flowgraphy 1 day after the intravitreal injection of anti-VEGF antibody immediately after the laser irradiation. (A) Representative images and the quantitative data of the retinal thickness in normal, vehicle, and anti-VEGF antibody-treated groups. The retinal thickness is significantly increased in the vehicle group, and this thickening was reduced by the injection of anti-VEGF antibody. (B) Color-coded laser speckle images from a representative eye from the vehicle- and anti-VEGF antibody-treated groups. The graph shows the quantitative data of ocular blood flow analysis of the MBR of the total area, the MBR of the vessel region, and the MBR of the tissue region. The blood flow is significantly reduced in the vehicle group, and this decrease is reduced by anti-VEGF antibody. (C, D) Changes in the retinal thickness and blood flow measured 1 day after the injection of anti-VEGF antibody and 7 days after the laser irradiation. (C) Representative images and the quantitative data of the retinal thickness in the normal, vehicle-treated, and anti-VEGF antibody-treated groups. The retinal thickness is significantly decreased in the vehicle group, and injection of the anti-VEGF antibody led to a decrease in the retinal thickness. (D) Color-coded laser speckle images and the quantitative data of ocular blood flow analysis from the representative in each group. The blood flow is significantly decreased in the vehicle-treated group, and the administration of anti-VEGF antibody caused a further decrease. Data are the means ± SEMs (n = 5). *P < 0.05, **P < 0.01 versus normal, *P < 0.05, **P < 0.01 versus vehicle (Student's t test).
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**References**

Pathophysiological Role of VEGF in RVO Murine Model


