Retina

Photocoagulation of the Retinal Nonperfusion Area Prevents the Expression of the Vascular Endothelial Growth Factor in an Animal Model

Makoto Gozawa, Yoshihiro Takamura, Seiji Miyake, Takehiro Matsumura, Masakazu Morioka, Yutaka Yamada, and Masaru Inatani

Department of Ophthalmology, Faculty of Medical Sciences, University of Fukui, Matsuoka, Eiheiji, Yoshida, Fukui, Japan

Correspondence: Yoshihiro Takamura, Department of Ophthalmology, Faculty of Medical Science, University of Fukui, 23-3 Shimoizukiko, Matsuoka, Eiheiji, Yoshida, Fukui, 910-1193, Japan; takamurayoshihiro@gmail.com.
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PURPOSE. The purpose of this study was to evaluate whether photocoagulation of the retinal nonperfusion area suppresses ocular vascular endothelial growth factor (VEGF) expression in a rabbit retinal vein occlusion (RVO) model.

METHODS. The retinas of pigmented rabbits were made ischemic by a laser on the main branch of retinal veins following intravenous injection of Rose Bengal. The eyes were enucleated before treatment and at 1, 7, and 14 days after laser occlusion. VEGF protein levels in the vitreous humor, sensory retina, and retinal pigment epithelium–choroid were measured with enzyme-linked immunosorbent assay. In situ hybridization of VEGF messenger RNA was performed to detect the location of VEGF expression in the sensory retina.

RESULTS. In the vitreous body, the VEGF protein level in the RVO group, but not that in the RVO + panretinal photocoagulation group, significantly increased on day 14. In the retina, the VEGF protein level in the RVO + panretinal photocoagulation group was significantly higher than that in the RVO group on day 1, but was significantly lower than that in the RVO group on days 7 and 14. In the in situ hybridization analysis, the RVO group showed a high expression of VEGF in the inner nuclear and ganglion cell layers on days 7 and 14. In contrast, VEGF expression in the RVO + panretinal photocoagulation group was strongly suppressed in both the inner nuclear and ganglion cell layers on days 7 and 14.

CONCLUSIONS. This study is the first using an animal RVO model to demonstrate that laser photocoagulation of the retinal nonperfusion area suppresses VEGF-A expression in the retina.

Keywords: retinal ischemia, laser photocoagulation, vascular endothelial growth factor

Retinal ischemia is a major cause of several eye diseases, such as diabetic retinopathy, retinal vein occlusion, and retinopathy of the premature. Vascular endothelial growth factor (VEGF) is a potent angiogenic and permeability-enhancing factor that plays an important role in these diseases.1,2 It is reported that a higher expression of messenger RNA (mRNA) of VEGF was observed in the ischemic retina than in the normal retina in patients3 and in an animal retinal ischemic model.3 VEGF derived from ischemic retina causes retinal neovascularization and results in vitreous hemorrhage, retinal detachment, neovascular glaucoma, and macular oedema, leading to severe visual disturbance. Therefore treatment that suppresses the expression of mRNA of VEGF and results in decreasing intraocular VEGF protein levels is important for improving visual acuity in retinal ischemic diseases.

Laser photocoagulation for retinal nonperfusion areas (NPAs) is a well-known technique for the treatment of retinal ischemic diseases.4–6 Peripheral scatter argon laser photocoagulation reportedly lessens neovascularization in treated eyes.7 Furthermore, our previous study showed that when compared with anti-VEGF therapy alone, targeting retinal photocoagulation to NPAs reduces the severity of macular edema recurrence after anti-VEGF therapy.8 However, no reports have used an animal RVO model to investigate whether photocoagulation of retinal NPAs suppresses the expression of VEGF.

Therefore, the aim of this study was to evaluate whether the laser photocoagulation of NPAs of the retina suppresses ocular VEGF expression in a rabbit RVO model.

METHODS

Animals

Animal experiments were conducted according to the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Use Committee of University of Fukui. A total of 21 female Japanese pigmented rabbits (2.0–3.0 kg, 12–15 weeks old) were used. For all procedures, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (Ketalar; 25 mg/kg body weight; Daiichi Sankyo, Tokyo, Japan) and xylazine hydrochloride (Celactal; 10 mg/kg body weight; Bayer Medical, Leverkusen, Germany). In the 42 eyes of 21 rabbits, we used 6 eyes of 3 rabbits without vein occlusion as controls. The rest of the 18 rabbits were equally divided into a RVO group and a RVO + panretinal photocoagulation (PRP) group. In each group,
RVO Creation and Laser Photocoagulation of NPAs

After the rabbits were anesthetized as described and their pupils were dilated with tropicamide and phenylephrine (Mydrin-P; Santen Pharmaceutical, Osaka, Japan) eye drops. RVO was created by laser occlusion of the retinal veins using an argon green laser (Iris Medical Oculight Gx; IRIDEX Corporation, Mountain View, CA, USA), as previously described. For the expected NPAs of RVO eyes, laser photocoagulation was performed (532 nm, 100 mW, 200 ms, 400 spots) simultaneously with RVO creation.

Color Fundus Photography and Fluorescein Angiography (FA)

The rabbits were anesthetized as described previously, and their pupils were dilated as for RVO creation. For FA, 0.5 mL of 10% sodium fluorescein (Alcon, Fort Worth, TX, USA) was injected intravenously into the marginal ear vein of the rabbits to obtain fluorescein angiograms between 6 seconds and 5 minutes after injection. FA was performed by experienced photographers with a Kowa VX-10i fundus camera (Kowa, Nagoya, Japan) to obtain fundus photographs from the entire region of the medullary wing. Color fundus photos were obtained using a slit lamp microscope through a Volk Super-Quad fundus lens (Volk Optical, Inc., Mentor, OH, USA) on the cornea using the same anesthesia protocol. The color fundus photographs were overlapped with the FA images in the same angle of view using Photoshop (Adobe Systems Incorporated, San Jose, CA, USA). We measured the area of the hemorrhage and nonperfusion and then calculated their ratio (%) for the original perfusion area of the same rabbits before RVO creation. We also compared the ratio of the hemorrhage and non-perfusion area in each group at days 1, 7, and 14.

ELISA for VEGF

On days 1, 7, and 14 after laser photocoagulation, the eyes were enucleated and the sensory retina and retinal pigment epithelium (RPE)-choroid complex were carefully isolated, placed in 150 μL of lysis buffer (20 mM imidazole hydrochloric acid (HCl), 10 mM potassium chloride (KCl), 1 mM magnesium chloride (MgCl₂), 10 mM ethyleneglycol-bis [beta-aminoethylether]-N, N'-tetraacetic acid, 1% Triton X-100, 10 mM NaF, 1 mM Na molybdate, and 1 mM ethylenediaminetetraacetic acid (EDTA); Sigma-Aldrich, Tokyo, Japan) and sonicated on ice for 15 seconds. The lysate was centrifuged at 14,000 rpm for 15 minutes at 4°C, and the VEGF levels in the supernatant were determined with a rabbit VEGF ELISA kit (detecting range 1.56–100 pg/mL; Cusabio Biotech Co. Ltd., Tokyo, Japan) at 450 to 570 nm and an absorption spectrophotometer (SpectraMax 34000A ROM, version 2.04; Bio-Rad, Hercules, CA, USA) and normalized to total protein according to the manufacturer’s protocol. A standard curve was plotted from the measurements of diluted standard solutions (7.8–500 pg/mL), and the concentration of VEGF in each sample was determined in comparison with this curve.

Total RNA Extraction and Complementary DNA (cDNA) Synthesis

Total RNA was isolated from rabbit retinas using a TRIzol reagent (Thermo Fisher Scientific, MA, USA) according to the manufacturer’s instructions. Purified total RNA was resuspended in deoxyribonuclease- and ribonuclease (RNase)-free water (Takara Bio Inc., Shiga, Japan) and stored at −80°C until use. The concentration of RNA was quantified using a NanoDrop 2000c (Thermo Fisher Scientific).

cDNA was synthesized from total RNA as the template using a ReverTra Ace qPCR RT Kit (Toyobo Co. Ltd., Osaka, Japan) according to the manufacturer’s instructions. This kit is optimized for the efficient synthesis of short-chain cDNA for real-time PCR experiments. However, because the primer mix included random and oligo dT primers optimized for efficient reverse transcription from all sizes of RNA, we adapted it as a cDNA synthesis for molecular cloning. The obtained cDNA was used as the template for the PCR reactions for gene cloning.

cDNA Cloning of the Partial Rabbit VEGF-A

A putative partial sequence of Dutch rabbit VEGF-A was predicted from a genomic database for Oryctolagus cuniculus genomes in Ensembl. An initial PCR amplification was carried out using KOD-plus (Toyobo Co. Ltd.), and the gene-specific primers (F1 and R1; Table 1) were designed according to the putative sequence. The PCR cycling parameters were as follows: an initial denaturation at 95°C for 2 minutes, followed by 30 cycles at 94°C for 15 seconds, 60°C for 30 seconds, and 68°C for 1.5 minutes, and storage at 10°C. In addition, nested PCR was carried out with the same cycling parameters using a combination of the reaction mixture from the initial PCR as a template and a second set of gene-specific primers (F2 and R2; see Table 1), which were also designed according to the putative sequence and located within the first primers.

After 1.5% agarose gel electrophoresis, the excised putative DNA fragment was extracted using a gel extraction kit (Qiagen N.V., Hilden, Germany) and cloned into the pGEM-T easy vector (Promega Corporation, WI, USA). After transformation into competent cells (Escherichia coli DH5α; Toyobo Co. Ltd.), the positive clones were sequenced in both directions using T7/SP6 primers and a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

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<th>TABLE 1. The Putative Gene Specific Primers for Rabbit VEGF-A</th>
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<td>First PCR</td>
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Tissue Fixative for Histochemical and Messenger RNA (mRNA) Expression Studies

In this study, Davidson’s fixative (strong formalin [37%), two parts; 95% ethanol, three parts; glacial acetic acid, one part; and distilled water, three parts) was used. This fixative is recommended for eye fixation particularly in the retina because it provides a morphological integrity superior to that of conventional formalin fixation. After enucleation, the eyes were exposed to fixative for 24 hours at room temperature and then immersed in 95% ethanol for 2 hours. For storage, tissues were transferred to 10% neutral buffered formalin until use. A paraffin infiltration of tissues was processed using Tissue-Tek VIP5 Jr. (Sakura Finetek Japan, Co. Ltd., Tokyo, Japan). Paraffin-embedded eyes (3–4 μm thick) were sectioned using a microtome (LS-115, Yamato Kohki Industrial Co. Ltd., Saitama, Japan).
Hematoxylin and Eosin Staining

The specimens were rehydrated and equilibrated with distilled water and then stained with hematoxylin (Carrazzi’s hematoxylin solution; Muto Pure Chemicals Co. Ltd., Tokyo, Japan.). For the removal of excess dye, differentiation was conducted in 70% ethanol containing 1% HCl. After tap water washing, the sections were dipped in 0.05% lithium carbonate solution followed by additional tap water washing to reveal nuclear details. The sections were then immersed in 70% ethanol before staining with eosin (1% Eosin Y solution; Muto Pure Chemicals Co. Ltd.) and washed with tap water. Finally, the steps for dehydration, clearing, and mounting were conducted using ethanol, xylene, and malinol (Muto Pure Chemicals Co. Ltd.), respectively.

In Situ Hybridization

All reactions were performed using RNase-free reagents and apparatus, and in situ hybridization was performed using a single-stranded RNA probe to enhance sensitivity and specificity. A purified plasmid DNA containing partial rabbit VEGF-A (Cosmo Bio Co. Ltd., Tokyo, Japan). Values are given as means ± standard errors. The Mann-Whitney U test was used for comparison of the ratio of hemorrhage and nonperfusion area. After the normal distribution of the data was confirmed, the VEGF concentrations at various time points were compared using the Tukey-Kramer honestly significant difference test. Differences with P values of <0.05 were considered statistically significant.

Results

Color Fundus Photography and FA

Figures 1 and 2 show the time courses of ocular fundus and FA images. On day 1 after RVO creation in the RVO group, color fundus photographs showed retinal hemorrhage along the medullary wings. On days 7 and 14, the retinal hemorrhage was decreased dramatically when compared with that on day 1, and chorioretinal atrophy became obvious, particularly on day 14. In the RVO + PRP group, the blocked vessels also resumed flow, but block of fluorescence owing to retinal hemorrhage and blocked blood flow. The vessels that were completely blocked on day 1 resumed flow on days 7 and 14, but the revascularized vessels did not reach to the peripheral region of the retina. In the RVO + PRP group, the blocked vessels also resumed flow, but did not reach to the peripheral region. Photocoagulation scars were observed in the NPA of the retina. Table 3 shows the comparison of the ratio of hemorrhage and nonperfusion areas.
hemorrhage, and nonperfusion areas, respectively. At all objective points, nonperfusion areas were significantly higher than hemorrhage areas.

**ELISA**

Table 2 and Figure 3 shows the VEGF protein levels in the vitreous body, retina, and RPE-choroid complex measured with ELISA. In the vitreous body, the VEGF protein level in the RVO group was significantly higher than that in the RVO + PRP group on day 14 (P = 0.047). In the retina, the VEGF protein level on days 7 and 14 in the RVO group was significantly higher than those in the control group (P < 0.0001 and P = 0.0015, respectively) and the RVO + PRP group (P < 0.0157 and P < 0.0079, respectively). When compared with the RVO group, the RVO + PRP group had significantly higher VEGF protein levels in the retina on day 1 (P = 0.0149). In the RPE-choroid, VEGF levels in the RVO + PRP group were significantly higher than those in the control and RVO groups on day 1 (P = 0.0427 and P = 0.0064, respectively).

**Hematoxylin and Eosin Staining**

Figure 4 shows that in the RVO + PRP group, the retina was disorganized, and chorioretinal adhesions were observed at photocoagulation spots on days 1, 7, and 14 after RVO creation. Replacement by the scar tissue and cell loss in the outer nuclear layer and retinal pigmentation became obvious as time passed. On the contrary, no remarkable differences were observed between the control and RVO groups after RVO creation. Retinal disarrangement, cell loss on each retinal layer, and changes in retinal thickness were absent.

**cDNA Cloning of the Partial Rabbit VEGF-A**

Molecular cloning of the putative rabbit VEGF-A was conducted to investigate the localization of the VEGF-A gene in the rabbit retina. For obtaining a cDNA clone of rabbit VEGF-A (O. cuniculus [Orcun]-VEGF-A), a nested PCR method was used with two combinations of gene-specific primers designed from a genomic sequence. This approach identified a single cDNA fragment (1121 bp), including a 494 bp portion of a putative open reading frame with 164 amino acids (Fig. 5). The obtained cDNA sequence covered approximately 77% of the putative open reading frame. The amino acid sequence of the cloned cDNA was identical except for a deletion of 24 serial amino acids (Fig. 6) when compared with the predicted amino acid sequence of O. cuniculus VEGF-A (XP_017200644.1). This deletion was identified as exon 6 (data not shown).

The presence of this gap was confirmed with a basic local alignment search tool (BLAST) search of the predicted VEGF-A sequences of other species at the same position (see Fig. 6). On the contrary, a protein BLAST analysis of the identified 164 amino acids indicated that the cloned VEGF-A had high identity with other cloned VEGF-A, such as boar (Sus scrofa, 95%), human (Homo sapiens, 91%), and cattle (Bos taurus, 93%) as well as rabbit (Ochotona curzoniae, 96%; see Fig. 7). The genome sequence was fully annotated, and the introns adjacent to exon 6 were consistent with the GT/AG processing rule. However, the tissue specificity of predicted sequence was not investigated. In the cDNA cloning from the rabbit retinas, randomly chosen positive clones lacked the insertion of 24 amino acids after transformation, which implied that the cloned VEGF-A was the predominant type in the rabbit retina. From these results, the cloned Orcun-VEGF-A was deemed likely to encode...
rabbit endogenous VEGF-A, and RNA probes were synthesized using this sequence as a template.

**DISCUSSION**

VEGF-A plays an important role in the pathogenesis of neovascularization and macular edema caused by retinal ischemic diseases. Clinically, laser photocoagulation of retinal NPAs is an established treatment for retinal ischemic diseases.4–6 Aiello et al. reported that intraocular levels of VEGF are remarkably high in the eyes of patients with active proliferative diabetic retinopathy, central retinal vein occlusion, and iris neovascularization, but those were extremely low in patients with quiescent proliferative diabetic retinopathy and iris neovascularization with a history of PRP14. In addition, an immunohistochemical study demonstrated that high expression of VEGF was observed in the inner retina and vascular endothelial cells of eyes with PDR, whereas this abnormal staining was not noticed in the retina of the eyes with quiescent PDR after PRP.2 Therefore, it is expected that laser photocoagulation contributes to reduce intraocular levels of VEGF in ischemic diseases; however, it has not been demonstrated yet. To the best of our knowledge, this report is the first to demonstrate, using an animal retinal ischemic model, that photocoagulation of retinal NPAs suppresses the expression levels of VEGF-A in the vitreous, sensory retina, and choroid.

Based on our data, the VEGF protein level in the sensory retina of the RVO group increased and peaked on day 7 and was still higher than that of the control on day 14. In the in situ hybridization (Fig. 8) analysis, the expression of VEGF-A in the ganglion and inner nuclear cell layers was low on day 1, it peaked on day 7, and was higher than that of the control on day 14. These results suggest that VEGF-A was derived from ganglion and inner nuclear cell layers in a laser-induced animal RVO model. This is not surprising because the circulation of the inner retina, including the ganglion and inner nuclear cell layers is maintained by the retinal circulation occluded by laser treatment. In addition, the production of VEGF-A from ganglion and inner nuclear cell layers may be able to explain that no signs of diabetic retinopathy developed in the retinal area with optic nerve atrophy in the patient with diabetes.15

The VEGF protein level in the RVO + PRP group was significantly lower than that in the RVO group on days 7 and 14. In the in situ hybridization (Fig. 8) analysis, the expression of VEGF-A in the ganglion and inner nuclear layer (INL) and ganglion cell layer (GCL) were significantly lower than that in the RVO group on days 7 and 14. The outer nuclear layer (ONL) that expresses VEGF-A under both nonischemic and ischemic conditions was replaced by the scar tissue after laser photocoagulation (Fig. 4). The expression of VEGF-A in the replaced area markedly decreased on days 7 and 14. This result suggests that photocoagulation of retinal NPAs suppressed the expression

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**FIGURE 3.** VEGF protein concentration in the vitreous humor (A), sensory retina (B), and RPE–choroid (C) measured with enzyme-linked immunosorbent assay. In the vitreous body, the VEGF protein level in the RVO group significantly increased when compared with that in the RVO + PRP group on day 14. In the sensory retina, the VEGF protein level in the RVO group was significantly higher than that of the control and RVO + PRP groups on days 7 and 14. The VEGF protein level in the sensory retina of the RVO + PRP group was significantly higher than that of the RVO group on day 1 (*P < 0.05, †P < 0.01).

**FIGURE 4.** Micrographs showing the histological differences in the sensory retina and RPE before and after RVO creation. Hematoxylin and eosin before RVO creation (A) and in the RVO + PRP group (B–D) and RVO group (E–G) on days 1 (B, E), 7 (C, F), and 14 (D, G). An asterisk indicates the photocoagulation site. NFL, neurofiber layer. Scale bar: 20 μm.
of VEGF-A from the ONL, INL, and GCL. In the ONL, the mechanism of lower expression of VEGF-A in the ONL of the RVOþPRP group was the loss of cells expressing VEGF-A replaced by the scar tissue. In the INL and GCL, the possible mechanism is not the cell loss but the improvement of hypoxia around the layers because remarkable cell loss in the INL and GCL was not observed in histological examination on days 1, 7, and 14 (Fig. 4). Stefánsson et al.4 investigated the physiologic mechanism of the photocoagulation of retinal NPAs in suppressing the expression of VEGF, hypothesizing that the physical light energy is absorbed in the RPE. The photoreceptors are destroyed and are replaced by a scar tissue, and oxygen consumption of the ONL is reduced. Now oxygen can diffuse through the scar tissue in the ONL without being consumed. This oxygen flux reaches the inner retinal layer to improve hypoxia and raise the oxygen tension, leading to the reduction of the production of VEGF.4 In the vitreous humor, the VEGF protein level of the RVO group was significantly higher than that of the RVOþPRP group, which suggests that VEGF protein accumulation was suppressed in the RVOþPRP group. Clinically, it is impossible to harvest not only the vitreous humor but also the retina, RPE, and choroid in humans; therefore, our study is valuable as it demonstrate that laser photocoagulation histologically suppresses the expression of VEGF-A of ischemic retina and lowers the intraocular VEGF protein level under the retinal ischemic condition.

The expression of VEGF-A in the sensory retina and the VEGF protein level of the sensory retina and the choroid in the RVOþPRP group was transiently higher than that in the RVO group on day 1. Our previous study using normal rabbits reported that retinal photocoagulation causes elevation of intraocular multiple cytokines, including VEGF protein, despite no retinal ischemia due to the inflammatory response to thermal burn, but this elevation is transient and can be suppressed by steroids or anti-VEGF therapy.16 The point is that the transient elevation of the VEGF protein level just after the photocoagulation can be suppressed by medical drugs, and...
Alignment between cloned rabbit VEGFA and predicted VEGFA

Cloned Oryctolagus cuniculus

Predicted Oryctolagus cuniculus

Ochotona curzoniae VEGF189

Orcinus orca

Physeter catodon

Manis javanica

Odobenus rosmarus divergens

PFMREGCDFKVFKMVFRYRSCQP1ET7.VDIFQEPYDEIEYIFKPSCVPLVRCCGCDNSELCEVPTEEFVNTQIM 80
PFMREGCDFKVFKMVFRYRSCQPIET7.VDIFQEPYDEIEYIFKPSCVPLVRCCGCDNSELCEVPTEEFVNTQIM 80
PFMREGCDFKVFKMVFRYRSCQP1ET7.VDIFQEPYDEIEYIFKPSCVPLVRCCGCDNSELCEVPTEEFVNTQIM 80
PFMREGCDFKVFKMVFRYRSCQP1ET7.VDIFQEPYDEIEYIFKPSCVPLVRCCGCDNSELCEVPTEEFVNTQIM 79
PFMREGCDFKVFKMVFRYRSCQP1ET7.VDIFQEPYDEIEYIFKPSCVPLVRCCGCDNSELCEVPTEEFVNTQIM 79
PFMREGCDFKVFKMVFRYRSCQP1ET7.VDIFQEPYDEIEYIFKPSCVPLVRCCGCDNSELCEVPTEEFVNTQIM 79
PFMREGCDFKVFKMVFRYRSCQP1ET7.VDIFQEPYDEIEYIFKPSCVPLVRCCGCDNSELCEVPTEEFVNTQIM 79
PFMREGCDFKVFKMVFRYRSCQP1ET7.VDIFQEPYDEIEYIFKPSCVPLVRCCGCDNSELCEVPTEEFVNTQIM 79

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Figure 6. Multiple alignment of the deduced amino acid sequence of Orcun-VEGF-A with several predicted VEGFα sequences. The solid line indicates the position of the gap. Conserved amino acids in all seven sequences are indicated by asterisks. The identities (%) against cloned rabbit VEGF-A are shown at the end of each sequence. The sequence identities of VEGF-A are predicted to be Oryctolagus cuniculus (XP_017200644.1), Ochotona curzoniae VEGF189 (ACA25170.1), Orcinus orca (XP_004267584.1), Physeter catodon (XP_007107652.1), Manis javanica (XP_017531751.1), and Odobenus rosmarus divergens (XP_012420569.1).
expression pattern of VEGF after the induction of retinal ischemia and the photocoagulation. Noninvasive observation of retinal tissue may contribute to further understanding of the relationship between the histological changes after vein occlusion and the degree of ischemia relating to the levels of VEGF. Optical coherence tomography, which provides a cross-sectional retinal image with high resolution, is used for not only clinical examination but also the experiments using animal models.9-20 However, it was difficult for us to detect a subtle change of retinal tissue after the venous occlusion (data not shown). In this study, therefore, we could not get more detailed findings using optical coherence tomography than histological examination and in situ hybridization.

In conclusion, our study is the first to demonstrate that the laser photocoagulation of retinal NPsAs suppresses VEGF-A expression in the sensory retina in an animal model. Laser photocoagulation is an effective treatment that decreases intraocular VEGF protein levels, leading to visual acuity in retinal ischemic diseases.

Acknowledgments
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