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

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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 group on day 1, but was significantly lower than that in the RVO group 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
nine eyes were used for ELISA to measure the levels of VEGF and the rest of the eyes were used for histological examinations. We used three eyes in each group at days 1, 7, and 14.

**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 Glx; 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-aminoethy-l ether]-N,N’-tetraacetic acid, 1% Triton X-100, 10 mM NaF, 1 mM Na molybdate, and 1 mM ethylenediaminetetraacetic acid with protease inhibitor; 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 (detection 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 resus-
Investigated using an established protocol for in situ hybridization solution (50% formamide, 5× saline sodium citrate [SSC], 200 µg/mL transfer RNA, and 50 µg/mL heparin) to a working solution of 50 ng/mL.

The distribution of VEGF mRNA in the rabbit retina was investigated using an established protocol for in situ hybridization12 in conjunction with the Roche DIG protocol to achieve a better signal-to-noise ratio. Paraffin-embedded sections (3–4 µm thick) were rehydrated via equilibration with diethyl pyrocarbonate (DEPC)-treated PBS and treated with proteinase K for 25 minutes at 37°C. The proteinase K-treated specimens were fixed with 4% paraformaldehyde in DEPC-treated PBS for 30 minutes. After equilibration with PBS, the dehydration was conducted through a graded methanol series to 100% methanol. The air-dried sections were hybridized with the probes at 60°C in airtight container (Incubation Chamber for 10 slides, High Temperature Light Shielding model; Cosmo Bio Co. Ltd., Tokyo, Japan).

For removal of excess probe after hybridization, two types of stringency washes were carried out at 60°C for 30 minutes using 2× SSC solution (25% formamide and 2× SSC in DEPC-treated water), followed by two washes in 0.2× SSC solution (25% formamide and 0.2× SSC in DEPC-treated water) at 60°C for 30 minutes. The specimens were then washed with maleic acid buffer (100 mM maleic acid buffer, 150 mM NaCl, and 0.1% Tween20) three times for 5 minutes each at room temperature. The detection of hybridized probes was performed according to the Roche DIG protocol. The specimens were maintained for 12 hours in the dark at room temperature. Color development was stopped with three washes of PBS, and the specimens were then fixed with 4% paraformaldehyde in PBS.

**Acquisition of Image Data**

The samples were observed under a microscope (IX70; Olympus Corporation, Tokyo, Japan) with a digital camera (DP73; Olympus Corporation). Image data were processed using Photoshop.

**Statistical Analyses**

Statistical analyses were performed by using JMP 10 (SAS Institute, Inc., 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, retinal hemorrhage and photocoagulation burns were observed away from the major vessels of the medullary wings. On days 7 and 14, retinal hemorrhage decreased and enlarged photocoagulation burns and chorioretinal atrophy were observed. FA images showed a 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. Photocoagulation scars were observed in the NPA of the retina. Table 3 shows the comparison of the ratio of hemorrhage and nonperfusion areas.
Surrounded with observed in the nonperfused area of the retina as black spots. Areas of the retina (*). In the RVO and 14, but the revascularized vessels did not reach the peripheral area chorioretinal atrophy were observed.

Hemorrhage decreased, and enlarged photocoagulation burns and scars as white spots were observed. On days 7 and 14, retinal hemorrhage along the medullary wings was observed on day 1. On days 7 and 14, the retinal hemorrhage was absorbed, and chorioretinal atrophy became obvious. In the RVO group, retinal hemorrhage and photocoagulation scars as white spots were observed. On days 7 and 14, retinal hemorrhage decreased, and enlarged photocoagulation burns and chorioretinal atrophy were observed.

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-As 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-As, such as boar (Sus scrofa), 95%; human (Homo sapiens, 91%), and cattle (Bos taurus, 93%) as well as rabbit (Occhotona curziorinae, 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. 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 PRP. 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. 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 analysis, the expression of VEGF-A in the ganglion and inner nuclear cell layers was 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...
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 RVO + PRP 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 RVO + PRP group, which suggests that VEGF protein accumulation was suppressed in the RVO + PRP 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 RVO + PRP 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

**FIGURE 5.** Nucleotide and predicted protein sequence of rabbit VEGF-A. The resulting complementary DNA (cDNA) sequence is 1121 bp with a partial open reading frame encoding 164 amino acids. Primers (F1 and R2) for cDNA amplification of the partial length of rabbit VEGF-A are underlined. The nucleotide sequence of the cloned Oryctolagus cuniculus (Orcun) VEGF-A was deposited in GenBank with accession no. LC310792.
the photocoagulation of retinal NPAs suppresses the expression and lowers the VEGF protein level in the longer term.

Although the retinal vessels of rabbits have some anatomical differences when compared with those of humans, the pathological effects of the rabbit RVO model is expected to be similar to humans. If the findings in our data using experimental models are confirmed in humans, a better way to treat vascular pathology of retina would be set in clinical practice. However, we are not able to obtain the retinal or choroidal tissue from the patients with vein occlusion. Also, we cannot obtain the vitreous sample repeatedly from same patient. Therefore, it is clinically difficult for us to monitor the the photocoagulation of retinal NPAs suppresses the expression and lowers the VEGF protein level in the longer term.

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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. 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 NPAs 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.

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References


