Nrf2 Activator RS9 Suppresses Pathological Ocular Angiogenesis and Hyperpermeability

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The work presented here and should therefore be regarded as equivalent authors.

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PURPOSE. Ocular angiogenesis, including retinopathy of prematurity, diabetic retinopathy, and exudative age-related macular degeneration, are closely related to oxidative stress. Many reports have shown that the cellular protective mechanism against oxidative stress and inflammatory response has nuclear factor-erythroid 2-related factor-2 (Nrf2) activity. The aim of this study was to investigate the effectiveness and mechanism of Nrf2 activation in treating the ocular diseases with abnormal vessels.

METHODS. The effects of Nrf2 activators, bardoxolone methyl (BARD) and RS9, were evaluated against vascular endothelial growth factor (VEGF)-induced cell migration in human retinal microvascular endothelial cells (HRMECs). We measured the expression of the Nrf2 target genes, Ho-1 and Nqo-1 mRNA, in mouse retinas after a single injection of BARD and RS9. The effects and mechanisms of RS9 against retinal angiogenesis were evaluated using an oxygen-induced retinopathy (OIR) model in mice. Moreover, the effect of RS9 against choroidal neovascularization (CNV) was evaluated in a laser-induced CNV monkey model.

RESULTS. Both BARD and RS9 decreased VEGF-induced cell migration, and significantly increased Ho-1 mRNA expression; however, only RS9 significantly increased Nqo-1 mRNA. RS9 decreased retinal neovascularization through suppressing VEGF expression and increasing Nrf2, HO-1, platelet-derived growth factor receptor (PDGFR)-β, and tight junction proteins in OIR murine retinas. Furthermore, RS9 showed a tendency toward decreasing CNV lesions, and improved vascular leakage in a CNV monkey model.

CONCLUSIONS. These data indicate that a Nrf2 activator might be a candidate for treatment of ocular diseases characterized by pathophysiological angiogenesis and hyperpermeability.
hemorrhage–induced brain injury,29,30 light-induced photoreceptor cell death,31 and Nalox-3-induced retinal pigment epithelium (RPE) cell damage.32

We hypothesized that the Nrf2 activation would inhibit ocular neovascularization and be effective for hyperpermeability through regulating antioxidant genes. The aim of the present study was to investigate the effect of RS9 against ocular angiogenesis and clarify the effect of Nrf2 activation in ocular angiogenesis.

MATERIALS AND METHODS

Cell Cultures

Primary human retinal microvascular endothelial cells (HRMECs) were obtained from DS Pharma Biomedical (Osaka, Japan). HRMECs were maintained in a humidified atmosphere with 5.0% CO2 at 37°C, with Cell Systems Corporation (CSC) complete medium (DS Pharma Biomedical) including 100 U/mL penicillin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and 100 ng/mL streptomycin (Meiji Seika Pharma Co., Ltd.). The CSC complete medium (4Z-500-R; DS Pharma Biomedical) is identical to a CSC medium (4Z-3-500-R; DS Pharma Biomedical) containing 10% fetal bovine serum and culture boost growth factor. HRMECs were maintained by passage every 3 to 4 days, and the cells from passages 3 to 8 were used for the experiments.

Scratch Test

In this assay, HRMECs were seeded at a density of 4.0 × 10⁴ cells/well in 12-well plates and incubated at 37°C in a humidified atmosphere of 5.0% CO2 for 24 hours. Afterward, the culture medium was replaced by CSC medium (DS Pharma Biomedical) with 1% fetal bovine serum (FBS) but without growth factor and incubated for 6 hours. The HRMEC monolayer at the center of the well was scraped and the culture medium was replaced by CSC medium with 1% FBS again to remove the floating cells. Both RS9 and BARD were added to a final concentration of 0.1 μM, and incubated for 1 hour before adding VEGF (R&D Systems Inc., Minneapolis, MN, USA) at a final concentration of 10 ng/mL. Before and after 24-hour incubation, four photographs covering an area of 3.6 mm² in each well were taken with a charge-coupled device (CCD) camera (Olympus, Tokyo, Japan). In this assay, cell migration was determined by the number of cells that migrated into the scraped area.

Monolayer Permeability Assay

HRMECs were plated at a density of 1 × 10⁴ cells/well onto Transwell inserts (Hanging Cell Culture Inserts; pore size 0.4 μm, membrane area 0.35 cm²; Merck Millipore, Billerica, MA, USA). The culture medium was replaced every other day. After cells were confluent, RS9 (1–1000 nM) was exposed. One hour after RS9 treatment, VEGF (100 ng/mL) was added. Twenty-four hours after VEGF exposure, using INVIVOX 200, transendothelial electrical resistance (TER) was measured using an Epithelial Volt-Ohm Meter (Millicell ERS-2; Merck Millipore Co., Darmstadt, Germany) and a cup-shaped electrode (Endohm-6; World Precision Instruments Inc., Sarasota, FL, USA). The following formula was used to calculate tissue TER: tissue TER (Ω cm²) = (sample well resistance − blank well resistance) × monolayer area. This formula was used according to the operating manual.

Animals

The experimental designs and all procedures using mice were approved by the Animal Experimental Committee of Gifu Pharmaceutical University. C57BL/6j mice (Japan SLC, Shizuoka, Japan) were used. All mice were housed in a room with a 12-hour light/dark cycle and ad libitum access to food and water. Nine male cynomolgus monkeys (Macaca fascicularis) were used in this study, aged 34 to 35 years and weighing 2.61 to 4.70 kg. Monkeys were housed in a room maintained at 24°C to 29°C with 30% to 70% humidity, ventilation 15 times/hour, and artificial lighting for 12 hours (7:00 AM–7:00 PM) and provided ad libitum access to water and approximately 108 g solid food (HF Primate J 12G 5KJ; Purina Mills LLC, Gray Summit, MO, USA) daily. Study procedures using monkeys were approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. (Kagoshima, Japan), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. All investigations were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RNA Isolation

The expression level of the mRNA of Ho-1 and Nqo-1 genes was determined 6 hours after RS9 or BARD (AdooQ BioScience, Irvine, CA, USA) intraperitoneal injection. Mice were euthanized and the eyeballs were quickly removed. The retinas were carefully separated from the eyeballs and rapidly frozen in liquid nitrogen. RNA was isolated from the cells with a NucleoSpin RNA kit (Takara, Shiga, Japan) according to the manufacturer's protocol. The RNA concentrations were determined spectrophotometrically at 260 nm by NanoVue Plus (GE Healthcare Japan, Tokyo, Japan), and aligned to 25 ng/mL. The isolated RNAs were converted to first-strand cDNA at 1.75 ng/mL using a PrimeScript RT reagent kit (Perfect Real Time; Takara) according to the manufacturer's protocol.

Quantitative Real-Time PCR (qRT-PCR)

The mRNA expression level of the Ho-1 and Nqo1 genes was determined by quantitative real-time-PCR (qRT-PCR). SYBR Premix Ex Taq II (Takara) and a TP 8000 Thermal Cycler Dice Real Time system (Takara) were used. Primers used in this assay were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
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<tr>
<td>Ho-1</td>
<td>5′-TGTGTCGTCGTGAGCTCTGA-3′ (sense) and 5′-TTGCTGTTTAAGTTGCTCCAG-3′ (antisense)</td>
</tr>
<tr>
<td>Nqo1</td>
<td>5′-CAAGGGTAGATTTCCGTGCCAG-3′ (antisense) and 5′-AGCTTCAGACCCCTTTGCAATGG-3′ (sense)</td>
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Pathophysiologic Neovascularization in Oxygen-Induced Retinopathy (OIR) Model Mice

OIR mice were created as described previously in detail with some modification.59 Neonatal mice and their mothers were transferred to a custom-built chamber on postnatal day 7 (P7). In this chamber, they were exposed to an atmosphere of 75% O2 for 5 days with the oxygen level controlled by oxygen controller (PRO-OX 110; Rermo Bioinstruments Co., Redfield, SD, USA). Neonatal mice were returned to room air on P12, and then RS9 at a dose of 1 or 3 mg/kg or BARD at a dose of 1 or 10 mg/kg was intraperitoneally administered. On P17, mice were anesthetized with an intraperitoneal injection of 10 mg/kg sodium pentobarbital were given an injection of 1 mL phosphate-buffered saline (PBS) containing 20 mg/mL fluores-
Physiological Neovascularization in Normal Mice
C57BL/6 mice (Japan SLC) were mated, and we used neonatal mice. To visualize the retinal blood vessels, they were sampled as described in the "Pathophysiological Neovascularization in Oxygen-Induced Retinopathy (OIR) Model Mice" methods section. RS9 was given subcutaneously at 3 mg/kg once a day from P3 to P7. As for evaluating pathophysiological neovascularization, to evaluate retinal physiological angiogenesis, tube area length, and area of retinal blood vessels were quantified using the Angiogenesis Tube Formation module in MetaMorph.

Immunoblotting
Mice were euthanized by decapitation at P14. The eyes were enucleated, and the corneas and lenses were removed. Then, the eye cups with retinas were quickly frozen in dry ice. The eye cups were homogenized in cell lysis buffer with a homogenizer (Physocron; Microtec Co. Ltd., Chiba, Japan). The lysate was centrifuged at 12,000 rpm for 20 minutes, and the supernatant was used for the following studies. The protein concentration was measured by comparing it to known concentrations of bovine serum albumin using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). A mixture of equal parts of an aliquot of protein and sample buffer with 10% 2-mercaptoethanol was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated protein was then transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, MA, USA). For immunoblotting, the following primary antibodies were used: Nrf2 rabbit monoclonal antibody [1:1000 dilution (=127218; Cell Signaling Technology, Danvers, MA, USA)], HO-1 rabbit polyclonal antibody [1:1000 dilution (AB1284; Merck Millipore)], VEGF rabbit polyclonal antibody [1:1000 dilution (PC315; Merck Millipore)], platelet-derived growth factor receptor (PDGFR)-β mouse monoclonal antibody [1:200 dilution (sc-374573; Santa Cruz, Dallas, TX, USA)], vascular endothelial-cadherin (VE-cadherin) rabbit polyclonal antibody [1:1000 dilution (=25008; Cell Signaling Technology), zonula occcludens-1 (ZO-1) rabbit polyclonal antibody [1:200 dilution (sc-33725; Santa Cruz)], claudin-5 rabbit polyclonal antibody [1:200 dilution (sc-574221; Santa Cruz)], and β-actin mouse monoclonal antibody [1:2000 dilution (A2228; Sigma-Aldrich, Tokyo, Japan)]. The secondary antibodies were goat anti-rabbit HRP-conjugated (1:2000: Thermo Fisher Scientific, Waltham, MA, USA), and goat anti-mouse HRP-conjugated (1:2000: Thermo Fisher Scientific).

Intravitreal Injection to Monkeys
Polyacetic-co-glycolic acid (PLGA) is one of the extensively researched synthetic biodegradable polymers. A wide range of PLGA-based drug delivery systems have been reported for the treatment of various diseases. For intravitreal injection of RS9 to monkeys, RS9 was diluted with PLA-0020, a polylactide (20 kDa; Wako Pure Chemical Industries, Ltd., Osaka, Japan) at a ratio of 1:1 of RS9 to PLA-0020. The concentration of RS9 was 0.3 mM. Sustained-release formulation RS9 PLA0020 was intravitreally administrated four times, just after laser irradiation and 1, 3, and 5 weeks after laser irradiation. Before administration, the area surrounding the region of eye to be dosed was disinfected with isodine solution (Meiji Seika Pharma Co., Ltd.), and the eyelid to be dosed was disinfected with PAIDO Ophthalmic and Eye Washing Solution (Nihon Tenganyaku Kenkyusho Co., Ltd., Aichi, Japan). After pupil dilation and instilling 0.4% oxybuprocaine hydrochloride (Benoxil ophthalmic solution 0.4%; Santen Pharmaceutical Co., Ltd.) for intravitreal injection was performed on animals anesthetized by ketamine or a mixture of ketamine and xylocaine (on the day of ophthalmic examinations) using a 30-gauge needle and a disposable syringe. A topical antibiotic agent (Gravit ophthalmic solution 0.5%; Santen Pharmaceutical Co., Ltd.) was instilled before and after administration. For the control experiments, the same volume of saline with PLA-0020 was injected in the same manner. Both eyes of all animals received either RS9 PLA0020 or vehicle. A total of six eyes per group were used in the present study.

Optical Coherence Tomography (OCT)
OCT images were obtained 2, 3, 4, 5, 6, and 7 weeks after laser irradiation. Monkeys were anesthetized by an intramuscular injection of a mixture of ketamine hydrochloride and xylocaine. After pupil dilation, scanning laser ophthalmoscope (SLO) and cross-sectional OCT images of individual laser-induced CNV in monkeys were obtained using a spectral-domain OCT system (Heidelberg Engineering GmbH, Heidelberg, Germany).

Histologic Analysis
At 7 weeks after laser irradiation, all monkeys were anesthetized by an intravenous injection of sodium pentobarbital (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) solution (64.8 mg/mL, 0.4 mg/kg) into the radicular vein and euthanized by exsanguination, and the eyeball and optic nerve were removed. The eyeball and optic nerve were immersed and immobilized in Davidson fixative overnight. On the following day, the tissues were horizontally sectioned and fixed in 10% neutral buffered formalin. Sections of individual CNV lesions were prepared and stained with hematoxylin and eosin (H&E) and Masson’s trichrome. Moreover, to histologically identify a CNV lesion, positive reaction (HistoGreen stain) for factor VIII (Von Willebrand factor) was detected using a primary anti-human Von Willebrand factor antibody (Agilent Technologies, Inc., Santa Clara, CA, USA) and a secondary anti-mouse antibody mixture of ketamine hydrochloride (50 mg/mL, Supriya Life-science Ltd., Mumbai, India) and xylocaine (2% Celacat; Bayel Medical Ltd., Osaka, Japan). The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P ophthalmic solution, Santen Pharmaceutical Co., Ltd., Osaka, Japan). Laser photocoagulation (532 nm, 1000 mW, 0.1 s, 80 μm) was applied to eight areas of the macula, but not the fovea, in both eyes of nine monkeys with a multicolor scan laser photocoagulator (MC-500; NIDEK Co. Ltd., Aichi, Japan).

Nrf2 Controls the Ocular Vascular Disease Progression
earlier with some modification. Nine monkeys were anesthetized by an intramuscular injection (0.2 mL/kg) of a 7:1 mixture of ketamine hydrochloride (50 mg/mL, Supriya Life-science Ltd., Mumbai, India) and xylocaine (2% Celacat; Bayel Medical Ltd., Osaka, Japan). The pupils were dilated with 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P ophthalmic solution, Santen Pharmaceutical Co., Ltd., Osaka, Japan). Laser photocoagulation (532 nm, 1000 mW, 0.1 s, 80 μm) was applied to eight areas of the macula, but not the fovea, in both eyes of nine monkeys with a multicolor scan laser photocoagulator (MC-500; NIDEK Co. Ltd., Aichi, Japan).
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(Envision+System-HRP Labelled Polymer Anti-Mouse, Agilent Technologies, Inc.).

**Fluorescein Angiography (FA)**

FA was performed 2, 3, 4, 5, 6, and 7 weeks after laser irradiation. After taking OCT images, fluorescein (Fluoresceite Intravenous Injection 500 mg, Alcon Japan Ltd., Tokyo, Japan, 0.1 mL/kg, 0.1 mL/s) was administered into the cephalic vein of the forearm. Photographs of the fundus were taken at approximately 1, 3, and 5 minutes after the administration of fluorescein with a fundus camera (GENESIS-DF; Kowa Company Co., Ltd., Aichi, Japan), and the photographs were defined as early, midtransit, or late frame of FA, respectively. To assess vascular leakage at photocoagulated spots, the following criteria were used in accordance with the previous study: grade 1 = no hyperfluorescence; grade 2 = hyperfluorescence without leakage; grade 3 = hyperfluorescence early or midtransit, and late leakage; grade 4 = bright hyperfluorescence early or midtransit, with late leakage extending beyond the borders of the laser spot. Vascular leakage grading was performed by two operators (T.N. and N.H.) with randomization. The percent of total grade 4 lesions, which was considered change relevant to clinical symptoms, was regarded as the severity of vascular leakage. One eye in the control group and one eye in the RS9 PLA-0020 group were not evaluated due to incidental eyeball opacity from 4 to 7 weeks after laser irradiation, and the data for this eye were excluded from the evaluation.

**Statistical Analysis**

Data are presented as the means ± standard error of the mean. Statistical comparisons were made by Student’s t-test and 1-way ANOVA followed by Dunnett’s test, and 1-tailed χ² test. SPSS Statistics (IBM Corp., Armonk, NY, USA) software was used for the statistical analysis. A P value < 0.05 was considered statistically significant. All experiments involved blind evaluation of the analysis.

**RESULTS**

**RS9 Inhibited VEGF-Induced Cell Migration and Improved VEGF-Induced Hyperpermeability in HRMECs**

We elucidated the effect of RS9 and BARD against VEGF-induced cell migration in HRMECs. VEGF enhanced cell migration in HRMECs (Figs. 1A, 1B). However, treatments with 100 nM RS9 or 100 nM BARD significantly inhibited VEGF-induced cell migration (Figs. 1A, 1B). Moreover, we evaluated TEER values as a strong indicator of the integrity of the cellular barriers in HRMECs. VEGF significantly decreased the TEER value (Fig. 1C). Exposure to RS9 attenuated the VEGF-induced TEER decrease in a concentration-dependent manner (Fig. 1C).

**RS9 Reduced Retinal Pathological Neovascularization in the OIR Model**

To compare the Nrf2 activation abilities of RS9 and BARD, we investigated expression of Nrf2 target genes, Ho-1 and Nqo-1 mRNA, in normal murine retina 6 hours after injection. The treatment with RS9 at doses of 1 and 3 mg/kg significantly increased the expression level of Ho-1 mRNA, whereas BARD increased it only at a dose of 10 mg/kg (Fig. 2A). Moreover, treatment with RS9 at 1 and 3 mg/kg increased the expression level of Nqo-1 mRNA, whereas BARD at 10 mg/kg did not increase it (Fig. 2B). To determine the effect of RS9 against retinal pathological neovascularization, we evaluated neovascular area percentage as a parameter of retinal pathological neovascularization in the murine OIR model by the administration of RS9 (3 mg/kg) (Fig. 2C). Based on Chikaraishi et al., 33 nodes were shown as connected blobs (accumulating of fluorescein-conjugated dextran) with thicknesses exceeding the maximum width of the vessels. Regarding node area and avascular area, RS9 significantly decreased these (Figs. 2D, 2E). To investigate the toxicity and antiangiogenic effect of RS9 against normal vessels, we investigated the retinal blood vessel morphology in normal mice at P8 after the RS9 (3 mg/kg) injection from P3 to P7. The treatment with RS9 did not change the length and area of retinal microvasculature compared with the vehicle-treated group (Figs. 2F–H).

**RS9 Suppressed VEGF Expression and Enhanced Tight Junction Protein Expression**

To clarify the mechanisms of RS9 in retinal angiogenesis, we investigated the expression of Nrf2, HO-1, VEGF, PDGFR-β, VE-cadherin, ZO-1, and claudin-5. Expression of both Nrf2 and HO-1 was increased with treatment with RS9 by the administration of RS9 (3 mg/kg), while VEGF expression was decreased. PDGFR-β, a pericyte marker, was upregulated. In addition, VE-cadherin, ZO-1, and claudin-5, which are tight junction proteins, were upregulated by RS9 treatment (Fig. 3).

**RS9 Suppressed Vascular Leakage in the Laser-Induced CNV Monkey Model**

We confirmed laser-induced CNV lesions by OCT images and histologic analysis (Fig. 4A). In the SLO and OCT images, lesions due to laser irradiation were observed (Figs. 4B, a–d). Also in histologic findings for H&E staining (Figs. 4B, e, f), Masson trichrome staining (Figs. 4B, g, h), and factor VIII immunostaining (Figs. 4B, i, j), there was no change between vehicle (PLA0020) and RS9 PLA0020 (0.015 × 10^{-3} mmol/eye). In fact, quantitative data were not changed (data not shown). To determine the effect of a sustained-release formulation of RS9 PLA0020 against monkey CNV, we evaluated vascular leakage in FA images at 7 weeks after laser irradiation (Fig. 4C). The severity of vascular leakage was graded from 1 to 4. The effect of RS9 PLA0020 was mild but certainly decreased grade 4 and increased grade 3 compared to the vehicle group (Figs. 4D–G). These results suggest that RS9 could change grade 4 to grade 3. At 6 and 7 weeks after laser irradiation, intravitreal injections of RS9 PLA0020 significantly decreased the percentage of grade 4 lesions compared to the vehicle-treated group (Fig. 4G). These data are consistent with the inhibitory effect of RS9 for hyperpermeability.

**DISCUSSION**

Our results showed that the Nrf2 activator, RS9, inhibited VEGF-induced cell migration and protected against microvascular endothelial barrier dysfunction in HRMECs. Moreover, in the in vivo neovascular model, RS9 not only inhibited retinal neovascularization and vascular leakage but also decreased VEGF expression and increased tight junction proteins in the retina. Nrf2 is closely related to oxidative stress. 35 These changes due to Nrf2 activation may be responsible for preventing oxidative stress.

Nrf2 activation has been shown to alleviate many pathological mechanisms associated with age-related diseases, particularly neurodegenerative diseases. 36,37 In angiogenesis-related diseases, Nrf2 acts as both a positive and a negative...
In cancer, Nrf2 promotes cancer progression. In contrast, Nrf2 in retinas decreases pathological angiogenesis. Genetically, Nrf2 deletion disturbed vascular regrowth and increased pathological angiogenesis. The present study, we also clarified that Nrf2 activator decreased endothelial cell migration (Fig. 1) and pathological angiogenesis in the OIR model (Fig. 2). Tan et al. indicated that increasing the expression of Nrf2-regulated antioxidant genes improved ischemic retinopathy by attenuating capillary vasoobliteration and neovascularization in the OIR model. These results agree with previous reports on retinas. In Nrf2 knockout murine retina, proangiogenic factors and inflammatory factors are upregulated. Specifically, proangiogenic factors such as hypoxia-inducible factor (HIF)-1α, VEGF, semaphorin (SEMA) 6A, and Notch signaling are increased.

As shown in Figure 3, our data and these previous reports are not mutually exclusive. Moreover, another Nrf2 activator, dh404, decreased VEGF expression and improved glial cell expression in retinas. It has been reported that retinal microglia cells contributed to vascular angiogenesis and vasculopathy induced by relative hypoxia. Interestingly, RS9 has no influence on physiological neovascularization (Figs. 2F–H). Nrf2 activation might inhibit pathophysiological neovascularization without side effects. Our research group has previously reported that addition of RS9 significantly increased expression of Nrf2 in the nucleus of photoreceptors and showed no cytotoxicity. Although we did not examine the effect of RS9 on the normal retina in vivo, it can be estimated

**Figure 1.** RS9 inhibited VEGF-induced cell migration and decreased TEER value in HRMECs. (A) Representative images of the wounded monolayer of HRMECs are shown. (B) Quantitative data of the VEGF-induced cell migration assay are shown. Data are shown as mean ± SEM (n = 4). ##P < 0.01 vs. control group (Student’s t-test). *P < 0.05 vs. vehicle-treated group (ANOVA with Dunnett’s post hoc test). (C) Quantitative data of TEER are shown. Data are shown as mean ± SEM (n = 3). ##P < 0.01 vs. control group (Student’s t-test), *P < 0.05 vs. vehicle-treated group (ANOVA with Dunnett’s post hoc test).
FIGURE 2. The effect of RS9 on the OIR model. Quantitative data of mRNA expression are shown. The mRNA expression of Nrf2 target genes (A) *Ho-1* and (B) *Nqo-1* in murine retina 6 hours after intraperitoneal injection of RS9 and BARD was measured by RT-PCR. Data are shown as mean ± SEM (n = 5–7). *P* < 0.05, **P** < 0.01 vs. vehicle-treated group (ANOVA with Dunnett’s post hoc test). (C) Representative images of OIR in retinal whole mount at P17 are shown in (a–c). Each magnified image of the region framed by white square is shown in (d–f). White asterisk, avascular area; white arrowhead, node of pathological neovascularization. Quantitative results of node areas (D) and avascular areas (E) are shown. Data are shown as mean ± SEM (n = 3). *P* < 0.05 vs. vehicle-treated group (ANOVA with Dunnnett’s post hoc test). (F) Flat-mounted retinas in normal mice (P8) are shown. Quantitative analysis of retinal vascular lumen at P8 was performed using an imaging analyzer (the Angiogenesis Tube Formation module) on the entire retina; two parameters were measured, length (G) and area (H). Scale bars: 100 μm (Figs. 4C, 4F). Data are shown as mean ± SEM (n = 5 or 6). N.S.: statistically not significant (Student’s t-test).
from previous in vitro data that RS9 increases the expression of Nrf2 and does not show toxicity in the normal retina. Furthermore, the overexpression of Nrf2 by RS9 in the normal retina might show hyperpermeability and/or an angiogenesis-preventing effect in the retina. In fact, we confirmed the cell toxicity of RS in HRMECs (Supplementary Fig. S1). Based on our results, we speculated that Nrf2 activation normalized retinal angiogenic factors in pathological conditions, inhibited ocular neovascularization and hyperpermeability, and might also improve the expression and activation of retinal glial cells. These findings comprehensively indicate that Nrf2 activation could help improve symptoms.

Macular edema is a cardinal symptom in DR or exudative AMD patients.50–54 In the same way, under retinal angiogenesis including both OIR and CNV models, vascular hyperpermeability occurred.3,55–58 In this study, we confirmed that VEGF-induced hyperpermeability in HRMECs (Fig. 1C) and laser irradiation developed fluorescein leakage in the FA analysis in monkeys (Fig. 4). Indeed, RS9 attenuated hyperpermeability (Figs. 1C, 4). Enhancing expression of Nrf2-driven genes protected against the blood–brain barrier (BBB) breakdown.

**Figure 3.** RS9 reduced VEGF expression and improved tight junction protein expression in the OIR model. (A) The relative intensity is defined as the fold change of Nrf2, HO-1, VEGF, PDGFR-β, VE-cadherin, ZO-1, and claudin-5 divided by β-actin. RS9 treatment increased Nrf2 (A), HO-1 (B), PDGFR-β (D), VE-cadherin (E), ZO-1 (F), and claudin-5 (G) expression and reduced VEGF (C) expression compared with the vehicle treatment group. The results for (B-G) were obtained from the same gel. Data are the mean ± SEM (n = 7 or 8). *P < 0.05 vs. vehicle treatment group (Student’s t-test).
**FIGURE 4.** RS9 reduced severe vascular leakage in the monkey laser-induced choroidal neovascularization (CNV) model. (A) The protocol for the laser-induced CNV model in monkeys is shown. Representative images in the vehicle-treated group (a, c, e, g, i) and the RS9 PLA0020-treated group (b, d, f, h, j) are shown. Representative images are seen in (a, b). Scanning laser ophthalmoscope (SLO) images, optical coherence tomography (OCT) images, (e, f) hematoxylin-eosin (H&E) stain images; (g, h) Masson’s trichrome stain images; and (i, j) factor VIII immunostaining images. The area shown in the OCT images corresponds in position to the area indicated by the green line in the SLO images. Laser-induced CNVs are framed in (e–j). Scale bar: 100 μm (Figs. 4B, e–j). (C) Representative images of fluorescein fundus angiography are shown. Quantitative results of the percentage of total grade 1 (D), grade 2 (E), grade 3 (F), and grade 4 (G) lesions over time are shown. The severity of vascular leakage at 40 or 48 lesions was graded. *P < 0.05, **P < 0.01 vs. vehicle-treated group (1-tailed χ² test).
after brain injury. In our previous report, another Nrf2 activator, BARD, also suppressed BBB permeability and upregulated endothelial cells and tight junction proteins. Similarly, expression levels of tight junction proteins and pericytes were high in the RS9 treatment groups (Fig. 3). These findings indicated that Nrf2 activation could protect the blood-retinal barrier. It has been reported that the expression level of tight junction proteins is upregulated by Nrf2 activation via defending ROS or inflammation. Moreover, the upregulation of HO-1, one of the Nrf2 target genes, regulates tight junction protein expression. These previous reports are strongly supportive of our results. Taken together, Nrf2 activation increases expression of tight junction proteins and suppresses vascular hyperpermeability.

RS9 has an antiangiogenic effect in OIR model mice but no effect on CNV area in the laser-induced CNV monkey model. One possible explanation for this is that primates have homeostasis in relation to ischemia and inflammation in the eye compared to rodents. It is generally known that pharmacokinetics and pharmacological effects differ greatly depending on animal species. Large animal species differences in results. Since current devices are at the limit between primates and rodents could be anticipated to lead to large animal species differences in pharmacokinetics and pharmacological effects differ greatly depending on animal species. It is generally known that pharmacokinetics and pharmacological effects differ greatly depending on animal species. Large animal species differences in results. Since current devices are at the limit between primates and rodents could be anticipated to lead to differences in results. Since current devices are at the limit between primates and rodents could be anticipated to lead to differences in results. Since current devices are at the limit between primates and rodents could be anticipated to lead to differences in results.

In conclusion, our data strongly suggest that Nrf2 might be a target protein for diseases characterized by ocular pathological angiogenesis including ROP, DR, and exudative AMD. Thus, RS9 may become a candidate for normalizing ocular angiogenic pathologies.

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