Suppression of Laser-Induced Choroidal Neovascularization by Nontargeted siRNA

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PURPOSE. To investigate the effect of nontargeted siRNAs on vascular leakage and vascular endothelial growth factor (VEGF)-A expression in the development of choroidal neovascularization (CNV).

METHODS. Nontargeted siRNAs were 21-nt (nucleotides) siRNA-Luc (Luciferase) or 16-nt siRNA-Luc. Targeted 21-nt siRNA-Vegfa or phosphate-buffered saline (PBS) was used for comparison. Laser photocoagulation was used to induce CNV in wild-type C57BL/6J mice; 7 days later, vascular leakage was determined by fluorescein angiography, and CNV volumes were measured by confocal microscopy. Expression of VEGF-A in the retinal pigment epithelium (RPE)/choroid was quantified by ELISA 3 days after photocoagulation.

RESULTS. Pathologically significant leakage developed in most of the 16nt-siRNA-Luc–or PBS-injected mice but in significantly fewer 21nt-siRNA-Luc– and 21nt-siRNA-Vegfa–injected mice (P = 0.0004, P = 0.0001, respectively). CNV volume in 21nt siRNA-Luc– and 21nt-siRNA-Vegfa–injected eyes was significantly lower than in PBS-injected eyes (P = 0.0124, P = 0.0004, respectively). CNV volume was not suppressed by 16nt-siRNA-Luc injection (P = 0.7700). The mean VEGF protein level decreased significantly in the 21nt-siRNA-Luc– and 21nt-siRNA-Vegfa–injected eyes compared with PBS-injected eyes 3 days after laser photocoagulation (P = 0.0011, P = 0.0003, respectively). The 16nt-siRNA-Luc–injected eyes did not show VEGF suppression 3 days after laser photocoagulation (P = 0.3177). Between 21nt siRNA-Luc– and 21nt-siRNA-Vegfa–injected eyes, there were no significant differences in CNV volume, the VEGF-A level, or pathologic leakage detected by fluorescein.

CONCLUSIONS. These data suggest that nontarget 21nt-siRNA can suppress laser-induced choroidal neovascularization anatomically and functionally through VEGF suppression. (Invest Ophthalmol Vis Sci. 2010;51:3820–3824) DOI:10.1167/iovs.09-5121

Age-related macular degeneration (AMD) is a leading cause of blindness around the world1-2 and is as common as cancer in many industrialized nations. Blindness in AMD results predominantly from invasion of the retina by choroidal neovascularization (CNV). Targeting the proangiogenic cytokine vascular endothelial growth factor (VEGF)-A has been validated in patients with CNV.3 Anti-VEGF-A antibody therapy is the standard of care for CNV,4,5 but this treatment is suboptimal: most patients do not experience significant visual improvement, repetitive injections are required for indefinite periods, and potential toxicity and immunologic concerns have emerged.6,7

Among the various proposed strategies, small interfering RNAs (siRNAs) have attracted much attention as a new therapeutic platform for achieving target-specific gene silencing through double-stranded RNA (dsRNA)–mediated RNA interference (RNAi). Clinical trials of naked VEGF-A siRNA (bevacizumab) or VEGFR1 siRNA (AGN211745/siRNA-027) were based on reports that these drugs suppressed laser-injury-induced CNV in a mouse model.8,9

It is interesting that these siRNAs were unformulated for cell penetration and yet exert antiangiogenic effects. Indeed, the combined challenges of intracellular delivery and unintended “off-target” effects of siRNAs are formidable.10 The polyanionic nature and molecular weight of siRNAs impede their entry into mammalian cells.11-15 In addition, siRNAs have both sequence-specific14-16 and sequence-independent17-19 off-target effects. Two recent reports presented data that 21-nt or longer siRNAs suppress hemangiogenesis and lymphangiogenesis in mouse models of choroidal, corneal, and dermal neovascularization not by RNAi but by activating cell surface toll-like receptor-3 (TLR3) on blood endothelial cells or lymphatic endothelial cells in a sequence- and target-independent manner.18,20

TLRs constitute a family of innate immune receptors that recognize various pathogen-associated molecular patterns. TLR3 is a sensor of dsRNAs,21 such as those found in viral genomes or replication intermediates, that undergoes dimerization22-25 and phosphorylation26 to initiate a signaling cascade that can ultimately result in apoptotic cell death.27-30 TLR3 also has a signaling cascade that can activate IL-12 and IFN-γ, which causes VEGF suppression.31-33

In this study, we sought to replicate these reported findings and to further explore this newly defined intersection between angiogenesis and innate immunity. We determined whether the generic antihemangiogenic effects of siRNAs also suppressed vascular leakage and VEGF-A expression in the development of CNV.

We used an siRNA targeting the same sequence targeted by bevasiraniib in human VEGFA (hVegfa siRNA), complementary to human VEGFA but mismatched to mouse Vegfa at nucleotide position 11, critical for functional targeting,34 a 21-nt siRNA targeting the nonmammalian gene firefly luciferase (21nt-siRNA-Luc), and a 16-nt siRNA targeting Luc that has been shown to not activate TLR3.35

MATERIALS AND METHODS

Animals

Male wildtype C57BL/6 mice (Japan SLC, Shizuoka, Japan) between 6 and 8 weeks of age were used to minimize variability. For all proce-
dures, anesthesia was induced by intramuscular injection of 50 mg/kg ketamine HCl (Sankyo, Tokyo, Japan) and 10 mg/kg xylazine (Bayer, Tokyo, Japan), and pupils were dilated with topical 1% tropicamide (Santen, Osaka, Japan). The study protocol was approved by the Nagoya City University Animal Care and Use Committee. All animal experiments were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Reagents

Nontargeted siRNAs were 21-nt siRNA-Luc (firefly luciferase) or 16-nt siRNA-Luc. Targeted 21-nt siRNA-Vegfa or vehicle buffer (phosphate-buffered saline; PBS) was used for comparison. The sequences (5'→3') of the sense strand of the siRNAs (Dharmacon, Lafayette, CO) are as follows: 21-nt siRNA-Luc, UAAGGCUAUGAGAUADTdT; 16-nt siRNA-Luc, UAAGGCUAUGAGAdTdT; 21-nt siRNA-Vegfa, ACCUCAGCAAGCCGACGC dTdT. RNAs were deprotected and annealed according to the manufacturer's instructions.

Induction of CNV

Laser photocoagulation (532 nm, 200 mW, 100 ms, 100 μm; Elite; Lumenis, Salt Lake City, UT) was performed (volume studies, 4–6 spots/eye; protein analyses, 20 spots/eye) bilaterally in each animal on day 0 by one person masked to the drug group assignment. Laser spots were applied in a standard fashion around the optic nerve using a slit lamp delivery system and a coverslip as a contact lens. The morphologic end point of laser injury was the appearance of a cavitation bubble, which is thought to correlate with the disruption of Bruch's membrane.

Drug Injections

16nt-siRNA-Luc, 21nt-siRNA-Luc, 21nt-siRNA-Vegfa, or PBS was injected into the vitreous humor of each wild-type mouse with a 33-gauge, double-caliber needle (Ito Corporation, Tokyo, Japan) immediately after laser injury, as described previously.35

Fluorescein Angiography

Fluorescein angiography was performed by an operator masked to the identity of the animal with a fundus camera (VX-10; Kowa, Nagoya, Japan) at 1 week after laser photocoagulation. Photographs were captured with a 20-D lens in contact with the fundus camera lens, after intraperitonal injection of 0.1 mL of 1% fluorescein sodium (Alcon, Tokyo, Japan).

Two masked retina specialists not involved in laser photocoagulation or angiography evaluated the fluorescein angiograms at a single sitting. Lesions were graded on an ordinal scale based on the spatial and temporal evolution of fluorescein leakage as follows: 0: (nonleaky) = no leakage, faint hyperfluorescence, or mottled fluorescence without leakage; 1: questionable leakage (arrow) = hyperfluorescent lesion without progressive increase in size or intensity; 2: leaky = hyperfluorescence increasing in intensity but not in size, no definite leakage; 3: pathologically significant leakage = hyperfluorescence increasing in intensity and in size, definite leakage (Fig. 1).

CNV Volume

One week after laser injury, eyes were enucleated and fixed with 4% paraformaldehyde. Eyecups obtained by removing anterior segments were incubated with 0.5% fluorescein-isothiocyanate (FITC)-isolectin B4 (Vector Laboratories, Burlingame, CA). CNV was visualized with blue argon laser wavelength (488 nm) using a scanning laser confocal microscope (LSM 5 Pascal; Carl Zeiss Meditec GmbH, Oberkochen, Germany).

Horizontal optical sections (1-μm step) were obtained from the surface of the RPE-choroid-sclera complex. The deepest focal plane in which the surrounding choroidal vascular network connecting to the lesion could be identified was judged to be the floor of the lesion. Any vessel in the laser-treated area and superficial to this reference plane was judged as CNV. Images of each section were digitally stored. The area of CNV-related fluorescence was measured by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The summation of whole fluorescein area in each horizontal section was used as an index for the volume of CNV, as described previously.36–37 The average of the volume obtained from all four to six laser spots per eye were generated (n refers to number of eyes). Imaging was performed by an operator masked to treatment group assignment.

VEGF ELISA

A previous report shows the mean VEGF level in the RPE/choroid peaked 3 days after photocoagulation.38 To detect VEGF protein levels in the RPE/choroid lysates 3 days after the application of 20 laser spots, the eyes were enucleated and the RPE/choroid complex was sonicated in lysis buffer (20 mM imidazole HCl, 10 mM KCl, 1 mM MgCl2, 10 mM EGTA, 1% Triton X-100, 10 mM NaF, 1 mM Na molybdate, and 1 mM EDTA with protease inhibitor cocktail; Sigma-Aldrich, St. Louis, MO) on ice for 15 minutes. VEGF protein levels in the supernatant were determined by an ELISA kit (threshold of detection, 3 pg/mL; R&D Systems, Minneapolis, MN) that recognizes all splice variants from 450 to 570 nm (SpectraMax340; Molecular Devices, Sunnyvale, CA) and were normalized to total protein (Bio-Rad, Hercules, CA).

Statistical Analysis

All results were expressed as mean ± SEM. Values were processed for statistical analyses (Mann-Whitney U test). Differences were considered statistically significant at P < 0.05.

RESULTS

21nt-siRNAs Suppressed Vascular Leakage from CNV

At 1 week after laser photocoagulation, pathologically significant leakage (grade 3 lesions) developed in most of the PBS- or 16nt-siRNA-Luc–injected mice but in significantly fewer 21-nt siRNA-Luc (1.0 μg)– or siRNA-Vegfa (1.0 μg)–injected mice.
(P = 0.0004, P = 0.0001, respectively) (Fig. 2). There was no significant difference in leakage between nontargeted 21-nt siRNA-Luc (1.0 μg)– and siRNA-Vegfa (1.0 μg)–injected mice (P = 0.9251).

21nt-siRNAs Suppressed CNV Size

Single intraocular administration of either 21-nt siRNA-Luc or 21-nt siRNA-Vegfa suppressed laser-induced CNV in wild-type mice in a dose-dependent fashion. The volume of laser-induced CNV was significantly reduced in the mice treated with 21-nt siRNA-Luc (248,493.8 ± 82,224.2 μm³ for 1.0 μg; n = 6; P = 0.0040) and 21-nt siRNA-Vegfa (197,130.2 ± 82,527.0 μm³ for 1.0 μg; n = 7; P = 0.0040) compared with PBS (n = 6) (Figs. 3A, 3B). However, 16-nt siRNA-Luc did not suppress CNV (461,505.9 ± 115,597.1 μm³ for 1.0 μg; n = 7; P = 0.7700).

There was no significant difference in CNV size between nontargeted 21-nt siRNA-Luc (1.0 μg)– and siRNA-Vegfa (1.0 μg)–injected mice (P = 0.6249).

21nt-siRNAs Suppressed VEGF Expression

VEGF levels in the RPE/choroid at 3 days after laser injury showed significant increases in the PBS-injected mice (21.7 ± 2.8 pg VEGF/mg total protein, P = 0.0047).

Mean VEGF levels in the RPE/choroid, which peaked on day 3, were significantly reduced in the 21-nt siRNA-Luc–injected mice (62.3% ± 4.7%, P = 0.001) and in 21-nt siRNA-Vegfa–injected mice (50.7% ± 4.6%, P = 0.001) compared with the PBS-injected mice (Fig. 4).
VEGF levels were not significantly different between 16-nt siRNA-Luc-injected and PBS-injected mice (17.9 ± 1.1 pg VEGF/mg total protein, P = 0.1175). There was no significant difference in VEGF levels between nontargeted 21-nt siRNA-Luc- and siRNA-Vega-injected mice (P = 0.1531).

**DISCUSSION**

In our studies examining the effect of nontargeted siRNAs in a laser-induced CNV model, we discovered that nontargeted siRNAs and 21nt-siRNAs, but not 16nt-siRNA, can suppress vascular leakage, VEGF expression, and CNV size. In the pathogenesis of CNV, VEGF had been thought to play a pivotal role\(^4\)\(^-\)\(^6\) and to be a therapeutic target, which led to establishment of the current anti-VEGF therapy for AMD.\(^4\)\(^-\)\(^5\)\(^,\)\(^4\)\(^2\)

Vascular leakage from CNV enhanced immature vessel formation and VEGF expression. Fluorescein angiography of CNV is used to quantify treatment efficacy clinically and is known to correlate well with visual function. The preclinical trial of siRNA targeting human VEGF-A also used the inhibition of vascular leakage in primates to demonstrate the efficacy of the treatment.\(^4\)\(^3\)

In this study, we evaluated the effect of nontargeted siRNAs in angiogenesis with a murine laser-induced CNV model, and we used both anatomic (CNV volume) and functional (fluorescein angiography) metrics of measuring CNV to reinforce the fidelity of our findings. Both anatomic and functional evaluation indicated that nontargeted siRNAs and 21nt-siRNAs in general could suppress laser-induced CNV.

Kleinman et al.\(^1\) showed that nontargeted siRNAs could suppress CNV by TLR3, which has the signaling cascade through TRIF to IFN-α/β, IL-12, or IFN-γ. In their studies, the mechanism underlying the antiangiogenesis effect by nontargeted siRNAs did not use the IFN-α/β pathway but, rather, the IL-12 or IFN-γ pathway.\(^1\) This is interesting because IL-12 and IFN-γ also showed VEGF suppression,\(^3\)\(^1\)\(^-\)\(^3\) which may lead to an antiangiogenic effect.

Our results showed VEGF suppression by 21nt-nontargeted siRNAs but not by 16nt-nontargeted siRNA, and these results support previous reports by Kleinman et al.\(^1\) that 21nt or longer siRNAs would activate TLR3 and show antiangiogenic effects. However, there are also several reports that TLR3 activation upregulates VEGF expression in vitro.\(^4\)\(^4\)\(^,\)\(^4\)\(^5\)

Our experimental model focused not only on TLR3 activation but also on preexisting injury (laser) and siRNA. Preexisting injury would dramatically change the response from cell culture. Kleinman et al.\(^1\) reported that siLUC did not change VEGF mRNA expression on the RPE/choroid, but another group reported that a TLR3 agonist did not upregulate VEGF in murine macrophages.\(^0\) Our finding that VEGF-A protein level was downregulated by 21nt-nontargeted siRNAs suggested that there is posttranscriptional regulation of VEGF-A by siRNA in laser injury.

It is already known that laser injury causes infiltration of inflammatory cells and that infiltrated inflammatory cells contribute to the development of CNV.\(^4\)\(^7\) Inflammatory cells that infiltrate the choroid are considered to be the major source of VEGF-A in the laser-injury induced CNV model.\(^3\)\(^6\)

Recently, Nakada et al.\(^4\) reported that TLR3 stimulation activates sflt-1 production by trophoblast cells. sflt-1 is a potent antiangiogenic molecule.\(^4\)\(^9\) and it would be worthwhile to investigate its regulation in laser injury and siRNA administration.

TLR3 plays a critical role in mammalian innate immune response against viral attacks by recognizing double-stranded RNA (dsRNA) or its synthetic analog, polyinosinic-polycytidylic acid (poly I:C). This leads to the activation of MAP kinases and NF-κB, which results in the induction of type 1, interferons, and proinflammatory cytokines (VEGFA) to combat the viral infection.

Kleinman et al.\(^1\) showed that the mechanism underlying the antiangiogenic effects of nontargeted siRNAs did not involve the IFN-α/β (type 1 interferons) pathway but, rather, the IL-12 or IFN-γ pathway, therefore, proinflammatory cytokine induction might not have occurred.

In our studies examining the antiangiogenic effect of nontargeted 21nt-siRNAs in CNV development, we discovered that 21nt-nontargeted siRNAs could suppress not only CNV volume (anatomically), they could also suppress fluorescein leakage (functionally) and VEGF-A expression, best known for its pivotal role in CNV development. Collectively, these data demonstrate that nontargeted 21nt-siRNA can suppress laser-induced choroidal neovascularization anatomically and functionally through VEGF suppression.

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**References**


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