Growth Factors, Cytokines, Cell Cycle Molecules

Inhibition of Ocular Angiogenesis by siRNA Targeting Vascular Endothelial Growth Factor Pathway Genes

Therapeutic Strategy for Herpetic Stromal Keratitis

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Ocular neovascularization often results in vision impairment. Frequently vascular endothelial cell growth factors (VEGFs) are mainly responsible for the pathological neovascularization as in the case in neovascularization induced by CpG oligodeoxynucleotides and herpes simplex virus infection in this report. siRNAs targeting either VEGFA, VEGFR1, VEGFR2, or a mix of the three were shown to significantly inhibit neovascularization induced by CpG when given locally or systemically. The efficacy of systemic administration was facilitated by the use of a polymer delivery vehicle. Additional experiments showed a significant inhibitory effect of the siRNAs mix when given either locally or systemically in vehicle against herpes simplex virus-induced angiogenesis as well as against lesions of stromal keratitis. These results indicate that the use of VEGF pathway-specific siRNAs represents a useful therapy against neovascularization-related eye diseases. (*Am J Pathol* 2004, 165:2177–2185)

New and uncontrolled blood vessel development in the eye is a pivotal process in the pathogenesis of several ocular neovascularization diseases, including herpes simplex virus (HSV)-induced stromal keratitis (SK), diabetic retinopathy, and age-related macular degenera-

tion.¹ These ocular diseases may finally cause blindness and managing them therapeutically is problematic. With HSV, the commonest infectious cause of vision impairment and blindness in the western world,² neovascularization of the avascular cornea represents an essential step in its pathogenesis.³ Multiple molecules may be responsible for the HSV-induced angiogenesis and it remains unclear how the virus infection results in the induction of angiogenic factors.⁴ Recently, we demonstrated that HSV DNA that contains abundant potentially bioactive CpG-containing motifs,^{5,6} can induce the potent angiogenesis factor vascular endothelial growth factor (VEGF) and that neutralization of VEGF with antibody minimized HSV-induced angiogenesis.³ A convenient model was also established in which bioactive CpG-containing oligodeoxynucleotides (ODNs) were also shown to induce neovascularization via the induction of VEGF.^{7,8} This model is used in the present study to evaluate the therapeutic potential of RNA interference (RNAi) to suppress VEGF expression and responsiveness.

Gene silencing by RNAi represents a potential important approach for therapy as well as a rapid and reliable tool for gene discovery or gene validation.⁹ Currently, small interfering RNA (siRNA) has received modest use *in vivo*.¹⁰ To achieve high efficacy in siRNA-mediated therapy, it is critical not only to choose the correct gene target and to design appropriate siRNAs, but more importantly also to efficiently deliver siRNAs into specific tissues or cells *in vivo*.¹¹ A previous report from our group demonstrated that targeting the VEGF protein and its receptor was a successful means of inhibiting neovascularization

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associated with tumor growth.¹² Other reports have also shown success using siRNA to MMP-9 against tumor angiogenesis.¹³ In the present study, we have used siRNA targeting the VEGF pathway and shown that this system significantly inhibits ocular angiogenesis induced by bioactive CpG and HSV infection. The results of these studies have implication for the control of HSV-induced vision loss.

Materials and Methods

Reagents

Phosphorothioate ODNs were kindly provided by Dennis M. Klinman (Biologics Evaluation and Research, Food and Drug Administration, Washington, DC). The sequences of stimulatory ODNs used in this study were: 1466, TCAACGTTGA, and 1555, GCTAGACGTTAGCGT. Subsequent studies were performed using an equimolar mixture of ODNs 1466 and 1555.

Molecular Design of Gene Targets and siRNAs

Three mVEGF pathway factors, mVEGF A and two mVEGF receptors (mVEGFR1 and mVEGFR2), were targeted by RNAi. For each gene target, two target sequences were assigned at different locations on the same mRNA. siRNAs were designed correspondent to the above target sequences. These siRNAs were designed according to the guideline proposed by Tuschl.^{14,15} The designed siRNAs (duplexes of sense and anti-sense strands) were synthesized by Qiagen (Valencia, CA). All siRNAs were 21-nucleotides long double-stranded RNA oligos with a two-nucleotide (TT) overhang at the 3' end. The targeted sequences of mVEGFA were (a) AAGCCGTCCTGTGTGCCGCTG and (b) AACGATGAAGCCCTGGAGTGC. The targeted sequences of mVEGFR1 were (a) AAGTTAAAAGTGCCTGAAGTGC and (b) AAGCAGGCCAGACTCTCTTTC. The targeted sequences of mVEGFR2 were (a) AAGCTCAGCACACAGAAAGAC and (b) AATGCGGCGGTGGTGACAGTA. The synthesis of unrelated siRNA controls, two target sequences each for LacZ and firefly luciferase were used. They were LacZ (a) AACAGTTGCGCAGCCTGAATG and (b) AACTTAATCGCCTTGACAGAC, Luc (a) AAGCTATGAAACGATATGGGC and (b) AACCGCTGAGAGCAACTGCA. Subsequent studies were conducted using an equimolar mixture of a and b for individual siRNA.

Mice

Female BALB/c mice (H-2^d), 5 to 6 weeks old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed conventionally. All investigations followed guidelines of the Committee on the Care of Laboratory Animals Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee (Knoxville, TN) are fully accredited by the American Association of Laboratory Animal Care.

Virus

HSV-I strain RE (kindly provided by Dr. Robert Lausch, University of Alabama, Mobile, AL) was used in all of the procedures. Virus was grown in Vero cell monolayers (catalog no. CCL81; American Type Culture Collection, Manassas, VA), titrated, and stored in aliquots at -80°C until used.

In Vitro Efficacy of siRNA

To test the efficacy of RNAi *in vitro*, the following cell lines were used. RAW264.7 gamma NO (-) cells (CRL-2278, ATCC) were used to test the efficiency of siVEGFA-specific knockdown of VEGFA gene that is spontaneously expressed in these cells. The cells were plated in a six-well plate in RPMI with 10% fetal bovine serum overnight at 37°C in 5% CO₂. One day after cell plating, the cells were transfected with different concentrations of siVEGFA or siLuc (at 0, 0.1, 0.5, 1.0, or 2.0 µg/2 ml/well, respectively) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours later RNA from these cells was extracted for reverse transcriptase-polymerase chain reaction (RT-PCR) to detect VEGF (see RNA Extraction and RT-PCR).

SVR cells (CRL-2280, ATCC) were used to test the efficiency of siVEGFR1-specific knockdown of VEGFR1 gene that is constitutively expressed on these cells. The cells were plated in a six-well plate in Dulbecco's modified Eagle's medium with 5% fetal bovine serum overnight at 37°C in 5% CO₂. One day after cell plating, the cells were transfected with different concentrations of siVEGFR1 or siLuc (at 0, 0.1, 0.5, or 1.0 µg/2 ml/well, respectively) using Lipofectamine 2000. Forty-eight hours later RNA from these cells was extracted for RS-PCR to detect VEGFR1 (see RNA Extraction and RNA-Template-Specific PCR) (RS-PCR).

The 293 cells (CRL-1573, ATCC) were used to transfect with mVEGFR2-expressing plasmid for the detection of knockdown of exogenous mVEGFR2. The cells were plated in a six-well plate in Dulbecco's modified Eagle's medium with 5% fetal bovine serum overnight at 37°C in 5% CO₂. One day after cell plating, the cells were co-transfected with plasmid pCI-VEGFR2 (0.2 µg/2 ml/well) and siVEGFR2 (a, b, a + b), or siLuc (0, 0.1, 0.5, or 1.0 µg/well, respectively) using Lipofectamine 2000. Forty-eight hours later RNA from these cells was extracted for RS-PCR to detect VEGFR2 (see RNA Extraction and RS-PCR).

RNA Extraction and RT- or RS-PCR

At various time points after siRNA treatment, the cells were harvested and RNA was extracted by using the RNeasy Protect mini kit (Qiagen, Valencia, CA). In case of ocular infection, two corneas per time point were excised and transferred to RNAlater. Briefly, cells were lysed in RLT buffer (catalog no. 79216, Qiagen) and RNA was purified according to the manufacturer's instructions. RNase-free DNase set (Qiagen) was used to remove any

contaminating genomic DNA. Total cellular RNA (10 μ g/ml) was reversed-transcribed using oligo (dT) primers and reverse transcriptase (Promega, Madison, WI), according to protocols described previously.¹⁶ The cDNA was made by the reverse-transcription reaction incubated at 42°C for 90 minutes. The primer sequences for mVEGF RT-PCR were: 5'-GCGGGCTGCCTCGCAGTC-3' (sense) and 5'-TCACCGCCTTGGCTTGTCAC-3' (anti-sense). RT-PCR products were 644 bp for mVEGF164 and 512 bp for mVEGF120. The amplification profile was 94°C for 1 minute, 65°C for 1 minute, and 72°C for 15 minutes for 30 cycles. The PCR products were separated by 1% agarose gel electrophoresis.

The RS-PCR was also performed for detection of mRNA knockdown by siRNAs *in vitro*. Cytoplasmic RNA was isolated by RNAwiz (no. 9736; Ambion, Austin, TX) according to manufacturer's instructions with additional DNase treatment, and subjected to RS-PCR with specially designed primers. The mRNA-specific reverse primers for the RT reaction were all 47-mer oligos with the 5'-end 30-mer of unique sequence (called "TS1" sequence, indicated in uppercase below) linked to a 17-mer sequence unique for each mRNA molecule (in lower case below). They were (from 5' to 3'): 1) mVEGFR1 dn: GAACATCGATGACAAGCTTAGGTATC-GATAtagattgaagattccgc; 2) mVEGFR2dn: GAACATC-GATGACAAGCTTAGGTATCGATAggtcactgacagaggcg. The PCR assays for all of the tested genes, that follow the RT assay, used a same reverse primer, TS1: GAACATC-GATGACAAGCTTAGGTATCGATA. The forward primers for PCR were all 30-mer oligos, unique for each gene: mVEGFR1up, GTCAGCTGCTGGGACACCGCGGTCTT-GCCT and mVEGFR2up, GGCGCTGCTAGCTGTCGCT-CTGTGGTTCTG. The RT-PCR of housekeeping gene GAPDH was used as control for RNA amount used in RS-PCR. An oligo dT primer (19 mer) was used for RT assay of GAPDH. The primers used for the followed PCR were 20-mer oligos: GAPDHup, CCTGGTCACCA-GGGCTGCTT and GAPDHdn, CCAGCCTTCTCCATG-GTGGT.

Corneal Micropocket Assay

The corneal micropocket assay used in this study observed the general protocol of Kenyon and colleagues.¹⁷ Pellets for insertion into the cornea were made by combining known amounts of CpG ODNs, sucralfate (10 mg, Bulch Meditec, Vaerlose, Denmark), and hydron polymer in ethanol (120 mg/1 ml ethanol; Interferon Sciences, New Brunswick, NJ), and applying the mixture to a 15 $\times$ 15 mm² piece of synthetic mesh (Sefar America, Inc., Kansas City, MO). The mixture was allowed to air dry and fibers of the mesh were pulled apart, yielding pellets containing 1 μ g of CpG ODNs. The micropocket was made ~1 mm from the limbus under a stereomicroscope (Leica Microsystems, Wetzlar, Germany) (four eyes per group) and pellets containing CpG ODNs were inserted into the micropocket.

Angiogenesis was evaluated at days 4 and 7 after pellet implantation by using calipers (Biomedical Re-

search Instruments, Rockville, MD) with a stereomicroscope. The length of the neovessels originated from the limbal vessel ring toward the center of the cornea and the width of the neovessels presented in clock hours were measured.⁴ Each clock hours is equal to 30° at the circumstance. The angiogenic area was calculated according to the formula for an ellipse. $A = [(clock\ hours) \times 0.4 \times (vessel\ length\ in\ mm) \times \pi]/2$.

In Vivo Delivery of siRNA

For local delivery, siRNA (10 μ g/10 μ l per eye) was diluted in phosphate-buffered saline (PBS) and delivered subconjunctivally. The subconjunctival injections were given by a 32-gauge Hamilton syringe (Hamilton Co., Reno, NV) at 6 and 24 hours after CpG pellet implantation or days 1 and 3 after virus infection under deep anesthesia induced by Avertin (Pittman Moore, Mondelein, IL). siRNA was injected 2 mm behind the limbus. For systemic injection, siRNA (40 μ g/100 μ l per mice) was mixed with polymer (TargeTran) and delivered intravenously. The tail vein injections were given at 6 and 24 hours after CpG pellet implantation or days 1 and 3 after virus infection using a 32-gauge syringe.

Corneal HSV-1 Infection

Corneal infections of all mouse groups were conducted under deep anesthesia induced by Avertin, St. Louis, MO. The mice were scarified lightly on their corneas with a 30-gauge needle, and a 2- μ l drop containing 1×10^5 plaque-forming units (PFUs) of HSV-1 RE was applied to the eye and gently massaged with the eyelids (six mice per group).

Clinical Observations (HSK Severity and Angiogenic Scoring)

The eyes were examined on different days after infection for the development of clinical lesions by slit-lamp biomicroscopy (Kawa Company, Nagoya, Japan), and the clinical severity of keratitis of individually scored mice was recorded. The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing SK. The severity of angiogenesis was recorded as described previously.⁴ Briefly, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summed to derive the neovascularization index (range, 0 to 16) for each eye at a given time point.⁴

Quantitative Real-Time PCR

Total cellular RNA was isolated from two corneas at day 7 after infection using the RNeasy RNA extraction kit (Qiagen) according to manufacturer's protocol. DNase

treatment (Qiagen) was done to remove any contaminating genomic DNA. To generate cDNA, 1 μ g of total RNA was reverse-transcribed using murine leukemia virus reverse transcriptase (Life Technologies, Bethesda, MD) with oligo(dT) as primers (Invitrogen). All cDNA samples were aliquoted and stored at -20°C until further use.

Real-time PCR was performed using a DNA Engine Opticon (MJ Research Inc., Cambridge, MA). PCR was performed using SYBR Green I reagent (Qiagen), according to the manufacturer's protocol. During the optimization procedures of the primers, 1% agarose gel analysis verified the amplification of one product of the predicted size with no primer-dimer bands. The absence of primer-dimer formation for each oligonucleotide set was also validated by establishing the melting curve profile. The semiquantitative comparison between samples was calculated as follows: the data were normalized by subtracting the difference of the threshold cycles (C_T) between the gene of interest's C_T and the housekeeping gene GAPDH's C_T (gene of interest C_T - GAPDH C_T = ΔC_T) for each sample. The ΔC_T was then compared to the expression levels of the vector control sample (sample ΔC_T - vector ΔC_T). To determine the relative enhanced expression of the gene of interest, the following calculation was made: fold change = $2^{(-\text{sample } \Delta C_T - \text{vector } \Delta C_T)}$. The primers used were murine GAPDH sense, CATCCTGCACCACCAACTGCTTAG and anti-sense, GCCTGCTTCACCACCTTCTTGATG; and murine VEGF164 sense, GCCAGCACATAGAGAGAATGAGC and anti-sense, CAA-GGCTCACAGTGATTTTCTGG.

VEGF Quantification of Corneal Lysates by Enzyme-Linked Immunosorbent Assay (ELISA)

The level of corneal VEGF protein expression was measured by a standard sandwich ELISA protocol. For preparation of corneal lysates, two corneas/time point ($n = 4$) were collected and minced. Minced pieces were collected in 1 ml of Dulbecco's modified Eagle's medium without FCS and homogenized using ultra sonicater (Heat systems-Ultrasonics, NY). The lysate was then clarified by centrifugation at 12,000 rpm for 5 minutes at 4°C . The supernatant was collected and stored at -80°C until further use.

The ELISA plate was coated with anti-mouse VEGF capture antibody (100 μ l/well of the capture antibody at 0.4 μ g/ml) and incubated at 4°C overnight. The plate was washed with 0.05% Tween 20/PBS and blocked with 3% bovine serum albumin for 2 hours at room temperature. After washing, serially diluted corneal lysates were added to the plate and incubated at 4°C overnight. The plate was washed and followed by anti-VEGF biotinylated detection Ab (R&D Systems, Minneapolis, MN) for 2 hours. Finally, peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratory, West Grove, PA) was added. The color reaction was developed using ABTS (Sigma-Aldrich, St. Louis, MO) and measured with an ELISA reader (Spectramax 340; Molecular Devices, Sunnyvale, CA) at 405 nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Figure 1. siRNA-mediated *in vitro* knockdown of VEGF pathway genes. RAW264.7 NO (-) cells (A) and SVR cells (B) in 35-mm wells were transfected with siRNA (equimolar mixture of two targeted sequences a + b) targeting mVEGFA and mVEGFR1, respectively, at the amount indicated. The 293 cells (C) were co-transfected with siRNA targeting mVEGFR2 (equimolar mixture of two targeted sequences a + b was used in subsequent experiments) and plasmid expressing mVEGFR2 at the amount indicated. Cellular RNA was isolated 24 hours (RT-PCR) or 48 hours (RS-PCR) after transfection, and the knockdown of endogenous expression of mVEGFA or mVEGFR1, or exogenous expression of mVEGFR2 was measured by RT-PCR for mVEGFA, or RS-PCR for mVEGFR1 and mVEGFR2.

Statistical Analysis

Significant differences between groups were evaluated by using the Student's *t*-test. $P < 0.05$ was regarded as significant difference between two groups.

Results

Knockdown of VEGF Pathway Genes by siRNA in Vitro

Three individual siRNAs were developed. These were siVEGFA, siVEGFR1, and siVEGFR2. Each was tested in a different cell system *in vitro* to measure its gene silencing efficiency. The siVEGFA was tested in RAW NO (-) macrophage cells, that produce VEGFA endogenously.¹⁸ Cells were transfected with siVEGFA, or control siLuc siRNA at various doses, and RT-PCR was performed 24 hours after transfection. As shown is Figure 1A, expression of both the 120 and 164 isoforms of VEGF was reduced by siVEGFA, in a dose-dependent manner. The efficacy of the siVEGFR1 reagent was evaluated in SVR cells that endogenously express the VEGF receptor 1.¹⁹ As shown in Figure 1B, VEGFR1 expression measured by RS-PCR 48 hours after transfection was diminished in a dose-dependent manner whereas all concentrations of

the control siLuc siRNA resulted in similar levels of VEGFR1 signal. Finally the siVEGFR2 reagent was tested in 293 cells that were exogenously transfected with a plasmid encoding VEGFR2. To measure the silencing effect of siVEGFR2, a co-transfection approach using siVEGFR2 and VEGFR2 DNA at various doses was performed. The 293 cells were co-transfected with various concentrations of siRNA targeting mVEGFR2 (0.1, 0.5, 1.0 $\mu\text{g}/\text{well}$) and plasmid expressing mVEGFR2 (0.2 $\mu\text{g}/\text{well}$). RNA was isolated 48 hours after co-transfection and exogenous expression of mVEGFR2 was measured by RS-PCR. Figure 1C shows that VEGFR2 expression was reduced by siVEGFR2, but not by the control siRNA molecule. These results indicate that all of the tested siRNAs were able to suppress the targeted genes *in vitro*.

Inhibition of CpG-Induced Angiogenesis by Local Delivery of siRNAs Targeting VEGF Pathway Genes

A previous study demonstrated that CpG-containing ODNs encapsulated in hydron pellets induce VEGF-mediated angiogenesis when inserted into corneal micropockets.⁷ This system was used to measure the inhibitory effect of local administration of siRNA preparations designed to target VEGF as well as two of its receptors (VEGFR1 and VEGFR2). A single dose of 10 μg of siRNA in PBS was used in all cases. This was administered by subconjunctival injection 24 hours after the establishment of micropockets containing CpG ODN. The siRNAs were tested individually as well as a 1:1:1 mixture of all three (siVEGFA, siVEGFR1, and siVEGFR2). New blood vessel formation in the corneal limbus was monitored at both days 4 and 7 after pellet implantation. As shown in Figure 2, significant inhibition of corneal neovascularization resulted with all three test siRNAs compared to those given control siLacZ at day 4 after pellet implantation ($P < 0.05$). The combination of the three tested siRNAs was the most effective inhibitor, providing an ~60% reduction in neovascularization ($P < 0.01$).

Inhibition of CpG-Induced Neovascularization by Systemic Delivery of siRNAs Targeting VEGF Pathway Genes

To test the anti-angiogenic effect of targeted individual siRNA and the efficiency of systemic siRNA delivery, mice with CpG ODN-containing micropockets were given a single dose intravenously of 40 μg siRNAs containing either siVEGFA, siVEGFR1, siVEGFR2, a mix of the three, or control siLacZ 6 and 24 hours after pellet implantation. In these experiments a polymer (TargetTran) was used that was shown in previous studies on tumor angiogenesis to facilitate extravascular delivery of siRNA.¹² At days 4 and 7 after pellet implantation, the extent of angiogenesis was measured. As shown in Figure 3, all reagents used individually induced significant inhibition of neovascularization compared to the siLacZ treated group at day 4 after pellet implantation ($P < 0.05$). As

Figure 2. Local delivery of siRNAs targeting VEGF pathway genes inhibits the CpG ODN-induced angiogenesis. Twenty-four hours after implantation with CpG ODN (1 μg) into the micropocket in mouse cornea, the mouse was given (10 μg per eye) siLacZ, siVEGFA, siVEGFR1, siVEGFR2, or siVEGF mix (an equimolar mixture of total siRNAs targeting VEGF pathway genes) by subconjunctival injection. The angiogenesis area was measured on days 4 and 7 after the CpG pellet implantation (four mice per group). **A:** Statistically significant differences in angiogenic areas (*, $P < 0.05$; **, $P < 0.01$) were observed between the groups. **B:** Images were taken by stereomicroscopic imaging system at day 7 after CpG pellet implantation. Original magnifications, $\times 40$.

observed with local administration, the mix of the three test reagents provided the most effective inhibition (average 40% inhibition, $P < 0.01$). In additional experiments, the function of the polymer vehicle was evaluated by comparing the anti-neovascularization activity of the test mix suspended in polymer or given in PBS. These experiments revealed that the use of the polymer vehicle resulted in more effective anti-neovascularization than was evident when the PBS vehicle was used although the result was only significant at the early test period ($P < 0.05$) (Figure 4A). The results demonstrate that ocular neovascularization can be controlled by the intravenous administration of siRNA that target the VEGF system genes and that the use of the TargetTran vehicle enhanced the efficacy of the therapeutic effect.

To determine the efficient anti-angiogenic dose of siRNA in systemic delivery, mice with CpG ODN-containing micropockets were given a single dose intravenously of 10, 20, 40, and 80 μg siRNAs containing a mix of the siVEGFA, siVEGFR1, and siVEGFR2, or control siLuc with TargetTran vehicle at 6 and 24 hours after pellet implantation. As shown in Figure 4B, administration of siRNAs

Figure 3. Systemic delivery of siRNAs against VEGF pathway genes inhibits the CpG ODN-induced angiogenesis. Individual siRNAs or a mixture of total siRNAs against VEGF pathway genes were delivered with TargeTran, 6 and 24 hours after the CpG ODN induction by tail vein injection. The angiogenesis area was measured on days 4 and 7 after the CpG pellet implantation (four mice per group). **A:** Statistically significant differences in angiogenic areas (*, $P < 0.05$; **, $P < 0.01$) were observed between the groups. **B:** Images were taken by stereomicroscopic imaging system at day 7 after CpG pellet implantation. Original magnifications, $\times 40$.

inhibited CpG-induced angiogenesis in a dose-dependent manner.

Therapeutic Application of siRNAs against VEGF Pathway Genes in the HSK Model

Previous studies have shown that VEGF is the critical angiogenic factor for induction of HSV-specific angiogenesis in the HSK model.³ To evaluate whether administration of siRNAs targeting VEGF pathway genes inhibits the development of HSK, the corneas of mice were scarified and infected with 1×10^5 HSV-1 RE. Then mice were given a single dose of 10 μ g (subconjunctival injection for local delivery) or 40 μ g (tail vein injection for systemic delivery) mix of siRNAs (an equimolar mixture of siVEGFA, siVEGFR1, and siVEGFR2) with polymer vehicle at days 1 and 3 after virus infection. As shown in Figure 5, the angiogenesis and severity of HSK was significantly reduced in mice treated with siRNAs targeting VEGF pathway genes either locally or systemically compared to animals treated with siLuc control ($P < 0.05$). Although 80% of siLuc control treated eyes developed clinically evident lesions (score 2 or greater at day 10 after infection), only 42% (local delivery) or 50% (sys-

Figure 4. Increased efficiency of siRNA systemic delivery by polymer and dose-response experiment. A mixture of siVEGF pathway genes or siLuc control (40 μ g) with polymer or PBS control was delivered 6 and 24 hours after the CpG ODN induction. The angiogenesis area was measured on days 4 and 7 after the CpG pellet implantation (four mice per group). **A:** Statistically significant differences in angiogenic areas (*, $P < 0.05$) were observed between the groups. Dose-response (10, 20, 40, and 80 μ g siRNA) experiment with mixed siRNAs targeting VEGF pathway genes with polymer in the systemic delivery system was performed. **B:** The angiogenesis area in each of the four mice for each group was measured on days 4 and 7 after implantation of CpG ODN, and the anti-angiogenic efficiency was compared between different siRNA dosages.

temic delivery) of eyes treated with siRNAs targeting VEGF pathway genes developed such lesions. In addition by day 10 after infection, the angiogenesis score was greater than 6 in 9 of 12 control eyes, but only in 5 of 12 eyes of mice treated with siRNAs against VEGF pathway genes by either local or systemic delivery. Taken together these results show that administration of siRNAs against VEGF pathway genes reduced development of HSK via inhibition of angiogenesis.

Decreased Level of VEGF mRNA after Treatment of siRNAs Targeting VEGF Pathway Genes in HSV-1-Infected Cornea

To address whether treatment of siRNAs against VEGF pathway genes reduces the level of VEGF mRNA, corneas were collected at days 4 or 7 after infection from mice that were infected with 1×10^5 PFU HSV-1 RE and were treated with siRNAs targeting VEGF pathway genes at days 1 and 3 after viral infection. The VEGF mRNA level

Figure 5. Reduced HSK severity and angiogenic response by administration of siRNAs targeting VEGF pathway genes. Mice were infected with 1×10^5 PFU HSV-1 RE per eye, and at days 1 and 3 after infection, were treated with siVEGF mix or siLuc with polymer either locally or systemically. Mean lesion HSK score (A) and angiogenic score (B) were calculated at day 10 after viral infection. Each dot represents the clinical score for one eye. Horizontal bars and figures in the parentheses indicate the mean for each group. Data are compiled from two separate experiments consisting of six eyes in each group. *, Statistically significant differences in HSK or angiogenesis score ($P < 0.05$) were observed between the groups. At day 14 after infection extensive growth of blood vessels and ulceration were seen in the infected cornea of siLuc-treated mice. C: siVEGF mix-treated mice showed less neovascularization near the limbal area.

was measured by RT-PCR or quantitative real-time PCR. As shown in Figure 6A, the expression of VEGF mRNA was reduced in the cornea treated with siRNAs against VEGF pathway genes compared to control eye at days 4 and 7 after infection. In addition, similar to what is found in RT-PCR, cornea treated with siRNAs against VEGF pathway genes showed the significant reduction in VEGF gene expression in comparison to cornea treated with siLuc control at 7 day after infection (Figure 6B).

Figure 6. Decreased level of VEGF mRNA in the cornea that was infected and treated with siVEGF mix with either local or systemic delivery. Two corneas were collected at days 4 or 7 after infection from mice that were infected with 1×10^5 PFU HSV-1 RE and were treated with siRNAs targeting VEGF pathway genes at days 1 and 3 after infection by either local (10 μ g, subconjunctival; S/C) or systemic (40 μ g, tail vein) administration and then VEGF mRNA level was measured by RT-PCR (A) or quantitative real-time PCR (B).

Decreased VEGF Protein Levels after Application of siRNAs against VEGF Pathway Genes in HSV-1-Infected Cornea

To evaluate whether treatment of siRNAs targeting VEGF pathway genes diminishes the production of VEGF protein, we measured VEGF protein in HSV-1-infected and siRNA-treated cornea at day 7 after infection. As shown in Figure 7, VEGF protein levels were lower in those that received siRNAs targeting VEGF pathway genes compared to controls given siLuc with polymer ($P < 0.05$). Once again administration of siRNA targeting VEGF pathway with polymer inhibited the production of its target gene in the HSK cornea.

Discussion

This study was undertaken to determine whether siRNAs targeting VEGF and VEGF receptor gene expression could effectively inhibit ocular vascularization. To measure the effect, angiogenesis was induced by inserting bioactive CpG into corneal micropockets, a system that may model the mechanism by which HSV-1 induces neovascularization and ultimately blindness.^{2,7,17} Our results show that siRNA administered either locally or administered systemically had significant inhibitory effects on the

Figure 7. Reduced levels of VEGF protein in the cornea that was infected and treated with siVEGF mix with either local or systemic delivery. At day 7 after infection two corneas per mouse were processed to measure the VEGF protein levels. Levels of VEGF were estimated from supernatants of corneal lysates of mice infected with HSV-1 and treated with siRNAs targeting VEGF pathway genes by an antibody capture ELISA as outlined in Materials and Methods. Results are expressed as mean $\pm$ SD of four separate mice (two corneas per mouse). *, Statistically significant differences in VEGF protein levels ($P < 0.05$) were observed between the groups.

neovascularization. Whereas individual siRNA targeted against VEGF or either of its two receptors had activity, maximal effects were observed when a 1:1:1 mix of the three siRNAs was used. Local administration to the conjunctiva was observed to result in the best therapeutic effect, but intravenous administration, especially when administered in the polymer vehicle TargeTran, was almost as effective. Additional experiments showed that the siRNA mixture given intravenously diminished VEGF protein levels in the HSV-1 infected cornea. The approach holds promise as a means to control ocular neovascularization, a pathological event in several ocular diseases.

Gene silencing by RNA interference has been well demonstrated to efficiently knockdown targeted genes *in vitro*, but few reports describe similar effects *in vivo*. One of the important factors for its reliability *in vivo* may be the delivery and transfection efficiency of siRNAs. However the efficiency of RNAi delivery systems in mammalian cells is still low.^{20,21} Based on previous reports, systemic delivery of siRNAs by tail vein injection could not efficiently deliver their siRNAs to their target areas because siRNA molecules are easily trapped in the nonspecific organs including liver, lungs, and spleen.^{11,22} The use of the polymer TargeTran may facilitate the delivery of contained reagents at inflammatory neovascular sites. Using a fluorescein isothiocyanate-labeled siRNA system we could demonstrate signals in the neovascular bed that were appreciably greater than observed when the labeled siRNA was administered in PBS. This polymer is composed of b-polyethylene imine (bPEI), polyethylene glycol, and arginine-glycine-aspartate peptide sequences (RGD).^{23,24} bPEI binds to negative charges in phosphates of the siRNA. RGD motif has been identified as an integrin ligand of activated endothelial cells.²⁵ Endothelial cells express a number of different integrins and integrin $\alpha v \beta 3$ and $\alpha 5 \beta 1$ have been shown to be important

during angiogenesis.^{25,26} Both integrins are the receptor for matrix proteins with an exposed RGD tripeptide moiety and are most prominent on cytokine-activated endothelial cells during angiogenesis.²⁵ Therefore in this study, we targeted the RGD-specific integrins on activated and proliferating endothelial cells for siRNA delivery. Our systemic delivery study showed that the angiogenic inhibitory efficiency of siRNAs with polymer was higher than that of siRNAs without polymer. In addition, we did not observe any abnormalities in mice that experienced systemic delivery of siRNAs with either polymer or PBS at least until the end of the experiment at 21 days after virus infection and siRNA treatment although VEGF pathway genes are necessary for physiological events. Because the half-life of siRNA molecules is short in serum, we believe not only that our siRNAs with polymer can specifically reach in the inflammatory area within short time, but also that the dosage of our siRNAs is not a toxic concentration in the mouse treated with siRNAs systemically.

Ocular neovascularization is abnormal proliferation and migration of new blood vessels from pre-existing vessel in the eye. Cornea is the avascular organ in normal eye.²⁷ However, if the cornea is infected or stimulated by angiogenic factors, the development of new blood vessels starts from the vessel of limbal area and newly formed blood vessels reach on the top of the cornea.²⁸ This neovascularization in the cornea supports the recruitment of inflammatory cells to the corneal tissue. These recruited inflammatory cells, especially neutrophils, produce many angiogenic factors such as VEGF and then these angiogenic factors induce angiogenesis and finally cause blindness. One of the diseases that show severe angiogenesis in the cornea is the herpetic stromal keratitis (HSK) induced by HSV-1.³ Recently several studies showed that potentially bioactive CpG-containing motifs of HSV DNA could stimulate cells to produce VEGF and induce angiogenesis in the eye.^{7,8} In addition, strong induction of VEGF expression was found in several neovascularized eye diseases. For these reasons, we designed our experiment using CpG ODN as angiogenic stimulator and siRNA against VEGF and its receptors as angiogenic inhibitor for the eye disease model.

In conclusion, using nanoparticle polymer systems, we delivered siRNA duplexes targeting VEGF pathway genes to inhibit the CpG ODN-induced corneal neovascularization with a mouse model. The significant inhibition of the CpG-induced angiogenesis observed in the study strongly suggested that the siRNA-mediated anti-angiogenesis effect represents a potential therapeutic for several uncontrolled angiogenesis diseases. In addition administration of siRNA duplexes targeting VEGF pathway significantly inhibits development of HSK. This present observation demonstrates that the use of VEGF pathway-specific siRNAs with clinically feasible delivery system holds great potential as a novel therapeutic for neovascularized eye diseases. It may also apply to other neovascularization-related eye diseases, including diabetic retinopathy and age-related macular degeneration.

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