Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model

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Purpose: RNA interference mediated by small interfering RNAs (siRNAs) is a powerful technology allowing the silencing of mammalian genes with great specificity and potency. The purpose of this study was to demonstrate the feasibility of RNA interference mediated by siRNA in retinal cells in vitro and in the murine retina in vivo.

Methods: siRNAs specific for enhanced green fluorescent protein (EGFP) and murine and human vascular endothelial growth factor (VEGF) were designed. In vitro studies in human cell lines entailed modulation of endogenous VEGF levels through chemically induced hypoxia. Effects of siRNA treatment on these levels were measured by ELISA. In vivo studies evaluating effects of siRNA on levels of EGFP and VEGF were performed by co-injecting recombinant viruses carrying EGFP or hVEGF cDNAs along with the appropriate siRNAs subretinally in mice. Additional studies aimed at blocking production of endogenous mVEGF were performed using laser-induced choroidal neovascularization (CNV) in mice. Effects of in vivo treatments were evaluated ophthalmoscopically. Retinal/choroidal flat mounts were evaluated after perfusion with dextran-fluorescein. Alternatively, retinas were evaluated in histological sections or VEGF levels were measured in intact eyes using ELISA.

Results: Successful delivery of siRNA to the subretinal space was confirmed by observing significantly reduced levels of EGFP in eyes treated with Ad.CMV.EGFP plus EGFP-directed siRNA. siRNAs directed against hVEGF effectively and specifically inhibit hypoxia-induced VEGF levels in human cell lines and after adenoviral induced hVEGF transgene expression in vivo. In addition, subretinal delivery of siRNA directed against murine Vegf significantly inhibited CNV after laser photoacoagulation.

Conclusions: Delivery of siRNA can be used in vitro and in vivo to target specific RNAs and to reduce the levels of the specific protein product in the targeted cells. This work suggests that RNA interference has potential for application to studies of retinal biology and for the treatment of a variety of retinal diseases, including those involving abnormal blood vessel growth.

RNA interference (RNAi) is a process conserved throughout many eukaryotic organisms, in which the presence of double stranded RNA (dsRNA) in a cell results in the destruction of mRNAs whose sequences share homology to the dsRNA. This phenomenon is now being exploited as a powerful tool for reverse genetics, and shows great promise for therapeutic applications. Elbashir et al. [1] have shown that synthetic RNAs of 21 and 22 nucleotides in length are able to mediate cleavage of the target RNA. These RNAs are termed small interfering RNA (siRNA). This group has also shown that siRNAs are involved in degrading homologous RNAs in mammalian cells [1]. Recently it was demonstrated that RNA interference mediated by siRNAs can specifically target sequence from hepatitis C virus in living mice [2,3].

Technology that allows the downregulation of expression of specific genes expressed in the retina would have numerous applications ranging from improving our understanding of the basic biology of the retina to providing therapy for blinding diseases. As a first step in evaluating the potential of retinal delivery of siRNA, we selected three target genes for study: the reporter gene, enhanced green fluorescent protein (EGFP) and both human (h) and murine (m) vascular endothelial growth factor (VEGF). We designed siRNAs directed specifically against these genes and tested their abilities to mediate RNA interference in vitro in human cell lines and in vivo in mice. The ability of siRNA molecules to silence endogenous VEGF gene expression was also evaluated in an animal model for age-related macular degeneration (AMD): the murine laser-induced model of choroidal neovascularization (CNV) [4]. CNV, found in the “wet” form of AMD, is characterized by the growth of new blood vessels below the retina. While multiple pro-angiogenic proteins may be involved in CNV, VEGF has been shown in both clinical and bench research studies to play a critical role in the pathophysiology of this blinding condition [5-14]. VEGF is known to be upregulated in the laser photoacoagulation model of CNV [15]. Our studies demonstrate that siRNA can be used to downregulate the expression of two different genes expressed in the retinal pigment epithelium (RPE). We also show that siRNA targeting Vegf can be used to decrease the extent of CNV in the laser photoacoagulation model. These findings suggest that delivery of siRNA directed against VEGF may be a useful approach with which to treat retinal diseases with a neovascular component.
METHODS

siRNA design: Selection of siRNAs was based on the characterization of siRNA by Elbashir et al [1,16]. An hVEGF5 siRNA targeting the sequence 5’-AAA CCU CAC CAA GGC GAC CAC-3’ was selected. This siRNA consisted of an RNA duplex containing a sense strand 5’-ACC UCA CCA AGG CCA GCA CtdTdT-3’ and an antisense strand 5’-UGC UGG CCU UGG UGA GGU TdT-3’. For rescue of the CNV model an siRNA was designed that targets mouse Vegf mRNA at the sequence 5’-AAC GAU GAA GCC CUG GAG UGC-3’. The sense strand of this molecule, mVegfl1 siRNA, was 5’-CGA UGA AGC CCU GGA GUG CtdTdT-3’ and the antisense strand was 5’-GCA CUC CAG GGC UUC AUC CtdTdT-3’. The siRNA used to downregulate EGFP expression targeted the following sequence in EGFP mRNA: 5’-GGC UAC GUC CAG CGC ACC-3’. The sense strand of this molecule, EGFP1 siRNA, was 5’-P GCC UAC GUC CAG CGC ACC-3’ and the antisense strand was 5’-P U GCG CUC CUG GAC GUU GCC UU-3’ (Pre-synthesized control siRNA green fluorescent protein duplex, Dharmacon Research Inc (Lafayette, CO)). The EGFP1 siRNA was also used as a control for studying the effects of hVEGF5 siRNA and mVegfl1 siRNA. All synthetic RNA sequences were synthesized and purified by Dharmacon Research, Inc.

Preparation of recombinant adenoviruses: Serotype 5, E1-deleted replication defective adenovirus vectors were prepared and used as described [17,18]. Transgenes were driven by the cytomegalovirus (CMV) promoter. The viruses were prepared in human embryonic kidney ‘293’ cells, purified by double CsCl density gradient centrifugation and stored in 10% glycerol at -70°C prior to use.

siRNA transfection and hypoxia induction in vitro: Human cell lines (human embryonic kidney ‘293’ and HeLa cells, ATCC, Manassas, VA) were seeded into 24 well plates one day prior to transfection. At the time of transfection with siRNA, the cells were about 50% confluent in 250 µl of complete DMEM medium. Transfections of siRNA (at 13 nM) were performed in all cells lines using the TransIT TKO Transfection reagent (Mirus, Madison, WI; using guidelines provided by the manufacturer). Controls included transfection reagent lacking siRNA, and nonspecific siRNA (EGFP1 siRNA). 24 h after transfection, hypoxia was induced in the cells by the addition of desferrioxamine to a final concentration of 130 mM in each well as described [19]. 48 h post transfection the supernatant was removed from all wells and a human VEGF ELISA (R & D systems, Minneapolis, MN) was performed on the cell supernatants as described in the Quantikine human VEGF ELISA protocol. ELISA results were read on a Wallac Victor2 ELISA plate reader (Perkin Elmer Life Sciences, Inc., Boston, MA). Experiments were repeated four times using 293 cells and three times using HeLa cells. The Student’s t-test was used for statistical analysis using the combined data from each of the replicates.

In vivo studies and tissue analysis: Animal experiments were performed in accordance with institutional guidelines for the care and use of animals in research. Both adenovirus and siRNA were delivered subretinally to five adult C57Bl/6 mice (Jackson Labs, Bar Harbor, ME) as described previously [17]. The mixture injected contained about 1x10^8 particles of Ad.CMV.EGFP, generously provided by Dr. J. Wilson (Dept. of Medical Genetics, University of Pennsylvania), and 20 picomoles of EGFP1 siRNA conjugated with transit TKO reagent (Mirus). As positive control, contralateral eyes received a mixture containing the same amount of Ad.CMV.EGFP and 20 picomolecules of hVEGF5 siRNA conjugated with transit TKO reagent (Mirus). Production of EGFP was detected by ophthalmoscopy 48-60 h after injection as described [20]. Animals were sacrificed and the eyes were enucleated and fixed in 4% paraformaldehyde. Eyes were prepared as either flat mounts (4 animals/8 eyes) or as 10 µm cryosections for fluorescent microscopy (1 animal/2 eyes). Microscopic examinations were performed with a Zeiss fluorescent dissecting microscope and with a Leica DMR microscope (Wetzlar, Germany) equipped with epifluorescence illumination. Images were captured using a Hamamatsu CCD camera (Hamamatsu Photonics, Bridgewater, NJ) using OpenLab 2.2 software (Improvision, Boston, MA).

Inhibition of human VEGF in vivo: Ad.CMV.hVEGF, generously provided by Dr. M. Herlyn (Molecular and Cellular Oncogenes, University of Pennsylvania), was injected subretinally and bilaterally in eyes of five C57Bl/6 mice. One eye of each animal was co-injected with mVegfl1 siRNA and contralateral eyes were co-injected with EGFP1 siRNA as control. Two days later, eyes were snap frozen in liquid N2, followed enucleation. All eyes were homogenized in lysis buffer (Roche, Basel, Switzerland) and total protein was measured using a Bradford assay. The samples were all normalized for total protein prior to assaying for human VEGF by ELISA. The ELISA was performed according to the manufacturers recommendations (R & D systems).

Laser induced CNV model studies: Adult (8-15 week old) female C57Bl/6 mice (n=30) were anesthetized with ativan (2,2,2-tribromoethanol) and pupils were dilated with 1% tropicamide. Laser photocoagulation was performed bilaterally using a diode laser photocoagulator (IRIS Medical, Mountain View, CA) and slit lamp system with a cover slip as a contact lens. Laser photocoagulation (140 mW, 75 µm spot size, 0.1 s duration) was applied to the 8 and 10 o’clock positions in the right eye and 2 and 4 o’clock positions in the left eye, 2 to 3 disk diameters from the optic nerve. Since the rupture of Bruch’s membrane is necessary to create significant CNV [4], bubble formation at the time of photocoagulation was used as an indication of the rupture of Bruch’s membrane. Laser burns that did not induce a rupture in Bruch’s membrane were excluded from the study. Lesions in which two laser spots became confluent were also excluded from the study.

Approximately 36 h after laser treatment, siRNA was delivered to both eyes by subretinal injection. A mixture containing about 1x10^8 particles of Ad.CMV.LacZ, generously provided by Dr. J. Wilson, and 20 picomolecules of mVegfl1 siRNA conjugated with transit TKO reagent (Mirus) was injected. As control, contralateral eyes received a mixture of the same
molecules except that mVEGF siRNA was replaced with EGFP1 siRNA. Two weeks after laser photocoagulation, animals were perfused with high molecular weight dextran-fluorescein (Molecular Probes, Eugene, OR) to label the retinal/choroidal vasculature and eyes were harvested. The area of CNV was measured in choroidal flat mount preparations by a masked individual using modifications of methods described previously [4,21]. These modifications are as follows: Microscopic examinations were performed with a Leica DMR microscope (Wetzlar, Germany) equipped with epifluorescence illumination. Lesions in the dextran-fluorescein-perfused flat mount preparations were identified as circular fluorescent (fluorescein positive) areas corresponding with the area previously exposed to the laser light. Images of the lesions were captured using a black and white Hamamatsu CCD camera (Hamamatsu Photonics, Bridgewater, NJ) coupled to a Apple Macintosh G4 computer (Cupertino, CA) equipped with OpenLab 2.2 software. Images for calibration were obtained from a slide with a grating of known size. The hyperfluorescent fluorescein-dextran labeled blood vessels within the area of the laser burn were selected as a “region of interest” (ROI) using Openlab software and this software was used to calculate the area (µm²) occupied by the white pixels in the ROIs. The ROIs were selected after collecting the images under identical integration settings. They were selected by using the Openlab “magic wand” tool to identify pixels in the laser burn site at a range of 2000-4090 intensity units. (The intensity units are defined within the Openlab software. The units selected represented levels measured in normal fluorescein-perfused vasculature. For reference, the intensity of background, non-fluorescent, areas was <450 intensity units.) The ROIs were generally well-circumscribed by a region lacking fluorescence. After measuring the areas of CNV, images were colorized in Openlab by applying an intensity ramp at 515 nm (the wavelength at which the image data were captured) using the “Apply wavelength” function. This intensity ramp was applied to all of the pixels in the image and made the whitest pixels the brightest green color. The images were then exported to Adobe Photoshop software for presentation purposes. Situations in which there was no evidence of a laser burn after bright field analysis of choroidal flatmounts were excluded. Statistical analysis of the results was performed using a one-tailed distribution, two sample unequal variance Student’s t-test.

RESULTS

Hypoxia-induced upregulation of human VEGF is halted by siRNA application in vitro: The ability of hVEGF5 siRNA to target human VEGF mRNA was tested in vitro in a system whereby exposure to desferrioxamine results in the induction of hypoxia-signaling events. That, in turn, induces VEGF expression [19].

Prior to hypoxia induction, two different human cell lines (embryonic kidney [293] cells and ovarian carcinoma [HeLa] cells) were transfected with hVEGF5 siRNA. As control, additional cells were transfected with an siRNA (EGFP1 siRNA) designed to target the reporter gene, EGFP, or with buffer alone. VEGF upregulation occurs due to a desferrioxamine-mediated induction of the HIF-1 hypoxic signaling pathway within 24 h [19]. To upregulate VEGF via this method, cells were exposed to desferrioxamine 24 h post-transfection. A human VEGF ELISA was performed on cell supernatants 24 h after desferrioxamine withdrawal in order to measure the effects of siRNA treatment on hVEGF levels. The hypoxia-mediated increase in hVEGF protein was reduced significantly in the presence of hVEGF5 siRNA (Figure 1). Exposure to the non-specific siRNA (EGFP1 siRNA) or to buffer lacking siRNA had minimal effect on hVEGF levels. EGFP1 siRNA

![Figure 1](http://www.molvis.org/molvis/v9/a31)
had no significant inhibitory effect on the concentration of hVEGF after exposure to hypoxic conditions in both 293 cells (Figure 1A; p=0.22) and HeLa cells (Figure 1B; p=0.15). There was no significant difference in hVEGF levels comparing normoxic and hypoxic, hVEGF5.siRNA-treated 293 cells (p=0.17). Human VEGF levels were significantly lower in the hypoxia plus hVEGF5.siRNA-treated cells than in cells treated with normoxia alone (p<0.01).

The ability to down-regulate VEGF production with an siRNA in vitro prompted us to explore the in vivo application of siRNA in the retina.

In vivo delivery of siRNAs to murine retinal pigment epithelial cells: Retinal pigment epithelial cells are thought to be the cells that, through abnormal VEGF production, initiate the process that leads to pathologic neovascularization (i.e. CNV) in AMD [12-14]. To determine whether siRNAs can be effectively delivered to the RPE, we expressed EGFP in those cells in vivo using a recombinant adenovirus, a virus that targets these cells efficiently and nearly exclusively after subretinal injection in adult mice. Recombinant adenovirus also results in a rapid onset of transgene expression [17,22]. In this system, we tested the possibility that EGFP1.siRNA would reduce levels of EGFP protein. Five mice received bilateral subretinal injections with Ad.CMV.EGFP, an E1/E3-deleted recombinant adenovirus delivering the EGFP cDNA driven by the CMV promoter. In the right eyes EGFP1.siRNA was also injected and in the contralateral eyes an siRNA unrelated to EGFP (hVEGF5.siRNA) was injected as a non-specific control. Ophthalmoscopy was performed 48 h post-injection, the time at which adenovirus-mediated transgene expression in this system is maximal [17]. Ophthalmoscopy in-
dicated that levels of EGFP were significantly lower in 4 of the 5 eyes co-injected with EGFP1.siRNA compared to the 5 eyes co-injected with hVEGF5.siRNA (not shown). The mice were sacrificed 48-60 h post-injection, and the eyes were harvested and evaluated for presence and amount of EGFP by inspection of retinal flat mounts and histological preparations. Figure 2A,B is representative of the flat mount results. There were only low levels of EGFP-specific fluorescence in whole mount preparations of the eyes that had received EGFP1.siRNA (Figure 2A) whereas EGFP was intensely fluorescent in retinas co-injected with hVEGF5.siRNA (Figure 2B). Uninjected portions of the eyes showed only background levels of fluorescence. Histological analyses revealed faint levels of EGFP in occasional cells of the injected portions of the retinas co-injected with EGFP1.siRNA (Figure 2C) but high levels of EGFP in the analogous regions of the contralateral eyes co-injected with hVEGF5.siRNA (Figure 2D).

Adenoviral expression of human VEGF silenced by siRNA in murine retina in vivo: Having demonstrated delivery of siRNA to RPE cells and significant diminution in levels of a marker protein, we sought to inhibit expression of a biologically relevant molecule in the retina in vivo. VEGF is an ideal candidate as this molecule is known to play a significant role in retinal neovascular disease [8-11]. Ad.CMV.hVEGF was used to deliver hVEGF as this virus can upregulate pharmacological levels of hVEGF and is capable of producing CNV when injected in the subretinal space [23,24].

One eye of each of 5 animals was co-injected with Ad.CMV.hVEGF and hVEGF5.siRNA while contralateral eyes were co-injected with Ad.CMV.hVEGF and EGFP1.siRNA as control. The animals were sacrificed 60 h post-injection and the eyes were processed for a human VEGF ELISA. There was a significant attenuation of VEGF levels in eyes that had received hVEGF5.siRNA as compared to the control

![Figure 4](http://www.molvis.org/molvis/v9/a31)

Figure 4. The extent of CNV is significantly reduced after subretinal delivery of mVegf1.siRNA. Thirty-six hours following laser photocoagulation, mVegf1.siRNA was delivered subretinally in one eye of each of 30 mice. Contralateral eyes were injected with a control (EGFP1) siRNA. Animals were perfused with dextran-fluorescein and the areas of CNV were measured in choroidal flat mounts 14 days after laser treatment. There is a significant difference (p<0.003) in mean areas of CNV between eyes injected with mVegf1.siRNA versus EGFP1.siRNA (panel A). Representative areas of CNV in eyes of a dextran-fluorescein-perfused animal that had received EGFP1.siRNA in one eye but mVegf1.siRNA in the other are shown in the colorized panels B and C, respectively. The CNV lesions were generally well-circumscribed by a region lacking fluorescence (as in panel B). CNV was identified by observing dextran-fluorescein-filled blood vessels on the choroidal/retinal interface, which are normally absent.
Inhibition of choroidal neovascularization using siRNA directed at murine Vegf: To determine whether siRNA can inhibit CNV in an animal model, we tested the effects of delivery of an siRNA directed against murine Vegf (mVegf1.siRNA) in a laser induced model of CNV. We delivered mVegf1.siRNA by subretinal injection to the RPE of mice 36 h after laser treatment. The areas of mVegf1.siRNA injection encompassed all of the laser spots as well as untreated retina adjacent to these spots. Contralateral eyes received siRNA targeting EGFP (EGFP1.siRNA) as control. The mice were perfused with dextran-fluorescein 14 days after the laser treatment, the time of maximal neovascularization, and the areas of neovascularization were measured using digital image capture around the burn spots. The majority of lesions (75% for the EGFP1.siRNA-treated eyes and 80% for the Vegf1.siRNA-treated eyes) met the criteria for analysis (see Methods). The locations of these neovascular areas exactly coincided with the sites initially exposed to laser. Neovascularization was not observed in portions of the retina that had not been exposed to laser. The areas of neovascularization in animals that received mVegf1.siRNA were, on average, one quarter of the area of the control-treated spots (Figure 4, p<0.003). These data show that siRNA targeting mVegf is capable of inhibiting CNV in the laser photoagulation model.

DISCUSSION

The data presented here represent the application of a technology to inhibit expression of genes in the retina, specifically to inhibit the expression of VEGF. To our knowledge, the data presented here describe the first successful application of siRNA to the retina. siRNA was used to significantly decrease levels of exogenous expression of transgenes both in vitro and in vivo. Results shown here from in vitro studies using siRNA directed against VEGF revealed a significant reduction in the amount of VEGF protein produced under hypoxic conditions. Even more impressive due to the high levels of protein produced after delivery of recombinant adenovirus, siRNA directed against VEGF significantly reduced VEGF protein levels after delivery of Ad.CMV.hVEGF in vivo. Finally, a test for relevance of VEGF-directed siRNA towards human retinal disease comes from studying its effect in an in vivo model of CNV. In accord with the in vitro and the in vivo Ad.CMV.hVEGF data, application of a VEGF-directed siRNA significantly reduced the extent of neovascularization in the murine laser photoagulation model of CNV. The results provide an encouraging first step in application of siRNA technology to the retina.

As with any new technology, the data from the siRNA studies presented here invite many questions. For example: how can this technology be applied to other (non RPE) retinal cell types? While these data demonstrate delivery of functional siRNA to the RPE they do not explore delivery of the molecules to other retinal cells. Subretinal delivery of adenovirus efficiently targets the RPE and occasional Müller cells, but not other layers of the adult retina [17]. Because the reporter protein was only expressed in the RPE we cannot currently assess the delivery of siRNA to other retinal cell types. We are currently employing other assays to address efficient delivery to the photoreceptors, Müller cells and ganglion cells. What is the stability of the effect? Is there any toxicity induced by the delivery or the composition of the siRNAs? Although there was no qualitative evidence of acute toxicity in the small number of samples we evaluated, it will be important to formally evaluate both acute and chronic toxicity of the treatment/siRNA in a larger number of eyes. In the case of laser photoagulation/CNV, how efficacious is the effect of VEGF:siRNA when it is administered at different stages of the disease? Will siRNA-mediated therapy be effective in other animal models of retinal neovascularization? VEGF siRNA-mediated rescue of additional models of ocular neovascularization such as the murine retinopathy of prematurity model [25] and models in larger animals are currently being studied in pursuit of answers to these questions.

The stability of the siRNA-induced interference effect on retinal cells is unknown at present. Experiments in progress aim to characterize the timecourse and magnitude of the effect after siRNA transfection. If this effect is found to be short-lived, there are other modifications that could be used to achieve a more sustained effect. For example, the combination of siRNA technology with recombinant viral vector techniques is a particularly promising avenue. Virus-mediated delivery of siRNA would allow delivery of therapeutic siRNA molecules that are replenished over time. Regardless of the mode of delivery, the potential of siRNA for contributing to a diverse set of applications is exciting. The possibilities range from using this technology to define developmental and physiological processes in the retina to testing approaches that might result in therapy for a diverse set of ocular diseases, including those involving ocular neovascularization.

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