Decrease of Murine Cytomegalovirus–Induced Retinitis by Intravenous Delivery of Immediate Early Protein-3–Specific siRNA

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Submitted: March 17, 2014
Accepted: May 21, 2014

Citation: Marshall B, Mo J, Covar J, Atherton SS, Zhang M. Decrease of murine cytomegalovirus–induced retinitis by intravenous delivery of immediate early protein-3–specific siRNA. *Invest Ophthalmol Vis Sci*. 2014;55:4151–4157. DOI:10.1167/iovs.14-14375

Purpose. Retinitis induced by both human and murine cytomegaloviruses following immunosuppression is characterized by progressive loss of retinal architecture, due to necrosis of virus-infected cells as well as widespread apoptosis of uninfected bystander cells. Because small inhibitory RNA molecules (siRNA) can reduce murine cytomegalovirus (MCMV) gene expression and thereby inhibit virus replication in vitro, we tested siRNAs directed against MCMV immediate early protein-3 (IE-3) to determine if MCMV-induced retinitis could be alleviated in vivo.

Methods. Immunosuppressed Balb/c mice (2.0 mg methylprednisolone acetate every 3 days beginning on day −2) were infected with 5×10^3 pfu of the K181 strain of MCMV via the supraciliary route. At day 2 post infection, mice were treated with various doses of IE-3–specific siRNA ranging from 0.1 nmol to 10 nmol, in a volume of 20 μL PBS via tail vein injection. Injected eyes were collected at various times post inoculation and subjected to plaque assay for virus titer, MCMV antigen staining, H&E staining, TUNEL assay, and Western blot for MCMV IE-3 protein.

Results. Small but significant amounts of fluorescently labeled IE-3–specific siRNA localized to the RPE layer 48 hours after intravenous injection. IE-3–specific siRNA significantly reduced virus titers at all concentrations tested (ranging from 0.1 nmol to 10 nmol), but the most potent effect of siRNA was observed at a dose of 1 nmol. We also observed that IE-3–specific siRNA produced a substantial decrease in MCMV titers and a substantial reduction in bystander cell apoptosis over the time course of virus infection.

Conclusions. Systemic administration of IE-3–specific siRNA could alleviate MCMV retinitis by inhibiting virus replication and subsequent death of uninfected retinal cells.

Keywords: siRNA, murine cytomegalovirus, retinitis, apoptosis

Retinitis and subsequent blindness induced by human cytomegalovirus has hitherto been a major complication of infection with human immunodeficiency virus (HIV), although with the advent of highly active antiretroviral therapy (HAART), this problem has abated considerably.[1–8] Human cytomegalovirus (HCMV) retinitis continues to be a chronic sight-threatening ophthalmologic problem among AIDS patients who do not respond to HAART or who discontinue therapy.[4–8] There is still a need for minimally invasive treatment protocols that not only inhibit ongoing cytomegalovirus (CMV) infections but that also can protect the retina from development of a destructive retinitis by inhibiting the initial events in CMV replication.[9]

Most of our current knowledge of CMV retinitis in AIDS patients is derived from sequential clinical observations and from microscopic examination of ocular tissues obtained post mortem. However, animal models of CMV retinitis have provided insights into the pathogenesis of HCMV retinitis. As CMVs are strictly species specific, HCMV cannot be studied experimentally in vivo. Hence, murine cytomegalovirus (MCMV) has been widely used as a model system to decipher the mechanism of CMV-induced pathology in human patients.[10,11] Our laboratory has used a mouse model in which injection of MCMV into the supraciliary space of immunosuppressed mice causes retinal infection with histopathologic features that mimic those observed in ocular specimens obtained from human patients. These features include apoptosis and necrosis of both virus-infected and -uninfected cells and infection of the RPE.[12–16] Our results have demonstrated that infection of the RPE layer is a critical early event in the disease process leading to MCMV spread to the inner and outer nuclear layers of the overlying retina several days later.[14–16] Virus-infected cells are protected from cell death by several MCMV-encoded proteins that inhibit both apoptosis and programmed necrosis (or necroptosis), allowing the virus to complete its replication program and produce abundant progeny.[17] These include the viral M45 (vIRA) protein, which inhibits activation of receptor-interacting protein 1 (RIP1) and RIP3 kinases and thus necroptosis,[18] as well as the viral M36 (vICA) protein, which inhibits caspase 8 activation.[19] Paradoxically, uninfected bystander cells located throughout the neural retina undergo cell death, leading to loss of overall retinal architecture and eventual blindness.[14] Extensive death of uninfected bystander cells is not uncommon during virus infections and is a significant cause of pathology.[20–25] Although the molecule or molecules responsible for bystander death in the MCMV-

infected retina have not been definitively identified, it is clear that virus infection is the trigger that initiates this process. Therefore, inhibition of MCMV replication in vivo could lead to reduced retinitis and improved architectural integrity of the infected retina. To test this possibility, we have attempted to identify inhibitors of MCMV replication in vitro and have previously shown that a small hairpin RNA (shRNA) directed against the MCMV immediate early protein-3 (IE-3) gene effectively inhibits virus replication in a host cell line.[26]

To effectively inhibit virus replication in vivo, small RNA molecules must accumulate in their target tissue in biologically meaningful amounts after administration in a minimally invasive manner. In this article, we have investigated the effect of anti-MCMV IE-3 small inhibitory RNAs (siRNAs) on virus replication in vivo. Our data demonstrate that, as a result of siRNA treatment, retinitis is substantially reduced in the eyes of immunosuppressed mice infected with MCMV.

Methods

Mice

Female euthymic BALB/c mice, 6 to 8 weeks old, were obtained from Taconic (Germantown, NY, USA). Animals were housed in accordance with National Institutes of Health guidelines. Mice were kept on a 12-hour light-dark cycle and given unrestricted access to food and water. All ocular injections were performed after the mice had been anesthetized with a mixture of 42.9 mg/mL ketamine, 8.57 mg/mL xylazine, and 1.43 mg/mL acepromazine at a dose of 0.5 to 0.7 mL/kg body weight. The treatment of animals in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Virus and Virus Titration

The original stock of MCMV (K181 strain) was a generous gift of Edward S. Mocarski (Stanford University School of Medicine, Stanford, CA, USA). Virus was prepared from the salivary glands of MCMV-infected BALB/c mice as described previously.[12] Virus stocks were titered by plaque assay on monolayers of M2-10B4 cells and stored at −70°C. A fresh aliquot of stock virus was thawed and diluted to the appropriate concentration immediately before each experiment.

Small Inhibitory RNA Treatment

The gene target sequence used for siRNA construction was as follows: IE-3-AACATAGATATTGTTACAGCA (MCMV genomic sequence GenBank Accession No. L06816, nucleotides 8072–8092).[27] An shRNA targeting the same sequence effectively inhibits virus replication in a host cell line.[26] An IE-3–negative control siRNA contained the same base composition as the above sequence but the order of nucleotides was scrambled. The green fluorescent FAM-labeled IE-3–specific siRNA was constructed by Ambion (Life Technologies, Grand Island, NY, USA). Regular IE-3–specific siRNA was constructed by Integrated DNA Technologies (Coralville, IA, USA).

Experimental Plan

Mice were immunosuppressed by intramuscular injection of 2.0 mg sterile methylprednisolone acetate suspension every 3 days beginning on day −2 and continued until euthanization. This treatment typically depletes 93% of the CD4+ and CD8+ T cells as well as macrophages from MCMV-infected mice as assayed by flow cytometry of splenocytes. Mice were injected with 5×10^3 pfu MCMV contained in a volume of 2 μL via the supraciliary route on day 0. On day 2 post infection, mice were treated with various doses of IE-3–specific siRNA ranging from 0.1 nmol to 10 nmol, in a volume of 20 μL PBS via tail vein injection. After euthanization, eyes were removed, homogenized in serum-free tissue culture medium using a handheld tissue homogenizer (Biospec Products, Inc., Racine, WI, USA), and plated on mouse embryonic fibroblast cells for detection of replicating virus. Eyes of additional mice were removed and prepared for MCMV antigen staining, hematoxylin and eosin (H&E) staining, TUNEL assay, and Western blot for MCMV IE-3 protein.

Immunofluorescence and TUNEL Assays

Eyes were frozen in optimum temperature cutting compound and 8-μM sections were cut in a microtome. Fluorescein-labeled rabbit anti-MCMV early antigen (EA)[28] was used at a dilution of 1:800. TUNEL assays were performed using the Promega TUNEL assay kit (Promega, Fitchburg, WI, USA). Slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with coverslips.

Retinitis Quantification

The method of retinitis quantification has previously been described elsewhere.[12] Briefly, virus-infected retinas were sectioned and stained with H&E and scored between 0 and 4, with 0 being normal, 1/2 = absence of cytomegaly and retinal folds involving less than three-quarters of the retinal section; 1 = absence of cytomegaly and retinal folds involving more than three-quarters of the retinal section; 2 = cytomegaly plus full-thickness necrosis in no more than one-quarter of the retinal section; 3 = full-thickness necrosis in more than one-quarter but not the entire retinal section; 4 = full-thickness necrosis in the entire retinal section.

Western Blots

Western blot analysis was performed as previously described.[29] Briefly, proteins were extracted from normal eyes and from MCMV-injected eyes or from medium-injected eyes. Equal amounts of protein were loaded for SDS-PAGE, followed by electroblotting onto polyvinylidene difluoride membrane (GE Healthcare, Piscataway, NJ, USA). After blocking with 5% nonfat dry milk for 1 hour at room temperature, membranes were incubated overnight at 4°C with primary antibody. Binding of horseradish peroxidase–conjugated secondary antibody was performed for 1 hour at room temperature. Immune complexes were detected by a chemiluminescence detection system (ECL; GE Healthcare) and exposure to x-ray film. To verify equal loading among different samples, the membrane was stained with anti-β-actin antibody. Rabbit anti-mouse cleaved caspase 3 (Cell Signaling, Danvers, MA, USA) was used at a dilution of 1:200. Rabbit anti-MCMV IE-3 has been described elsewhere[17] and was used at a dilution of 1:1000. Anti-rabbit secondary antibodies (Santa Cruz, Dallas, TX, USA) were used at a dilution of 1:1000.

Results

RPE Cells Are Targeted by siRNA Molecules

Because MCMV replication begins in the RPE layer of the mouse eye after supraciliary injection of virus, we wondered whether it would be possible to inhibit virus replication in vivo by using siRNA targeted to the MCMV IE-3 protein. To

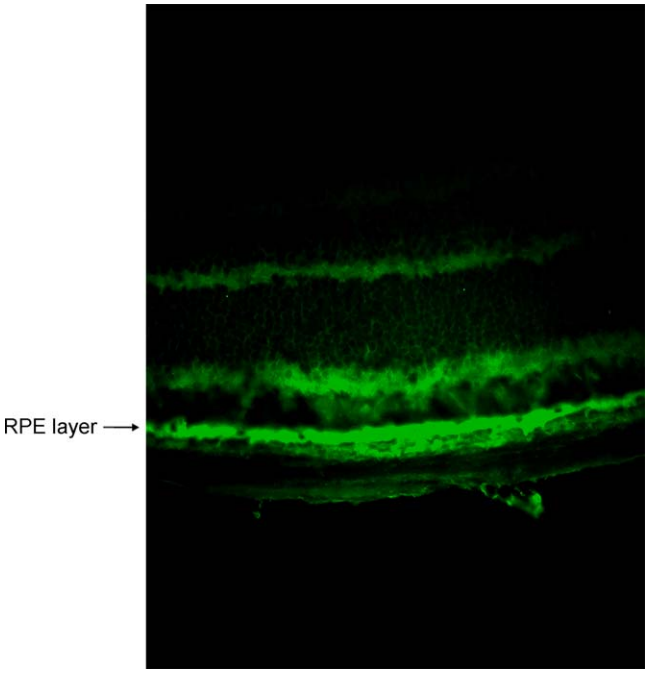

FIGURE 1. Photomicrograph of fluorescently labeled siRNA in the eye 48 hours after intravenous inoculation. Green fluorescence was detected throughout the RPE layer.

determine if the RPE layer of the eye could be targeted by siRNA molecules, we injected siRNA labeled with the green fluorescent FAM tag via the tail vein of uninfected mice and 2 days later harvested eyes for analysis. As can be seen in Figure 1, green fluorescence was detected throughout the RPE layer but not elsewhere in the eye 48 hours after injection,

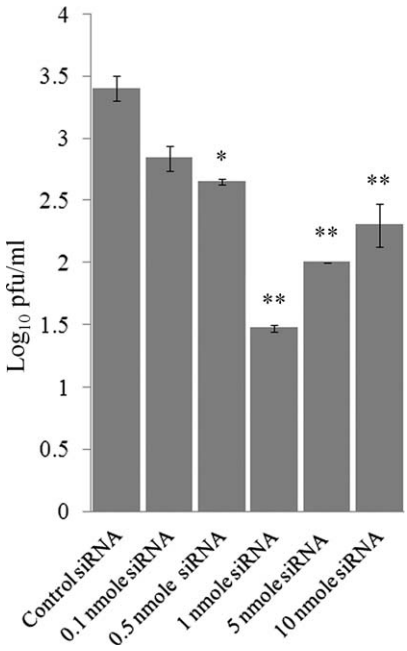

FIGURE 2. Virus titers in MCMV-injected eyes of immunosuppressed Balb/c mice at day 7 p.i. Indicated doses of anti–IE-3 siRNA or control siRNA were administrated at day 2 p.i. to groups of three mice per treatment. IE-3–specific siRNA significantly reduced virus titers (*$P <$ 0.02, **$P <$ 0.01). Statistical significance was calculated using a Student's *t*-test.

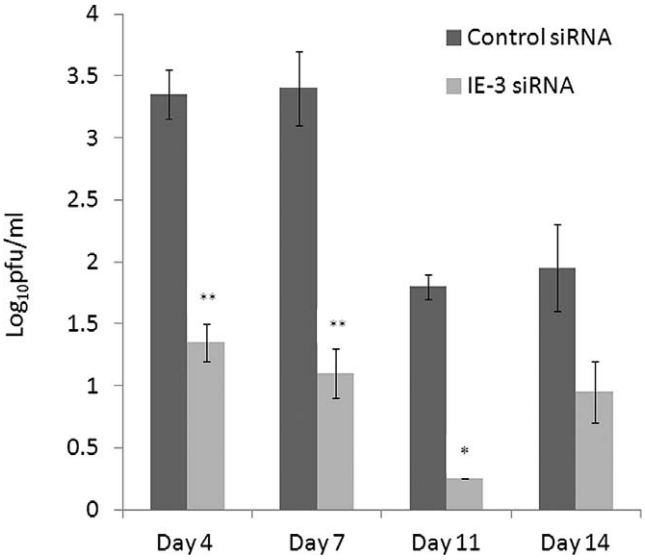

FIGURE 3. Virus growth curves after intraocular infection of immunosuppressed Balb/c mice with 5×10^3 pfu MCMV. Mice were treated with 1 nmol of either anti–IE-3 or control siRNA at day 2 p.i. IE-3–specific siRNA produced a substantial decrease in MCMV titers over the time course of virus infection, compared with control siRNA (*$P <$ 0.01, **$P <$ 0.001). Groups of three mice were used for each treatment and statistical significance was calculated using a Student's *t*-test.

indicating that at least some of the injected siRNA localizes to the eye. Most of the injected siRNA localizes to internal organs, such as the liver, kidney, and spleen (not shown), as previously reported.[30]

Optimum Dose of IE-3–Specific siRNA Providing the Maximum Inhibitory Effect on Virus Replication

To learn if the fluorescence present in the RPE layer corresponded to biologically active siRNA and to determine which dose would be most effective in inhibiting virus-induced retinitis, we tested several different doses of siRNA, ranging from 0.1 nmol to 10 nmol, for their effects on virus replication at day 7 post infection (p.i.) Immunosuppressed Balb/c mice were injected via the supraciliary route with 5×10^3 pfu of the K181 strain of MCMV, and several different doses of IE-3–specific siRNA ranging from 0.1 nmol to 10 nmol, were administered in a volume of 20 μL PBS via tail vein injection 48 hours later. Control mice received an injection of siRNA with a scrambled sequence. Mice were killed at day 7 p.i. and eyes harvested for plaque assay. BALB/c mice tolerated siRNA very well and no adverse symptoms were observed in any siRNA-treated mice. The IE-3–specific siRNA significantly reduced virus titers starting at a concentration of 0.5 nmol (Fig. 2), but we observed the most potent effect of siRNA at a dose of 1 nmol. Above 1 nmol there was no additional effect of siRNA as seen at treatment doses of both 5 and 10 nmol.

The Effect of IE-3–Specific siRNA on Virus Replication Over the Time Course of Virus Infection

To determine the effect of siRNA on virus replication over the time course of virus infection, MCMV-infected immunosuppressed Balb/c mice were intravenously injected with 1 nmol of either IE-3–specific or control siRNA 48 hours post-MCMV inoculation as described above, mice were killed at several

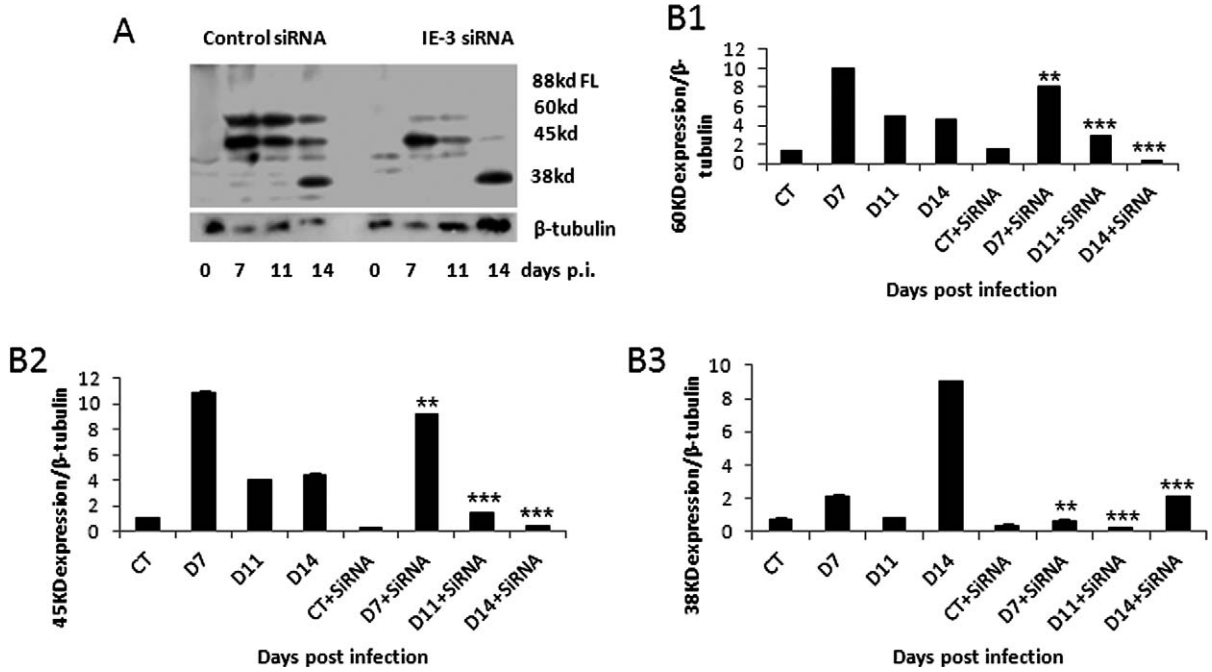

Figure 4. (**A**) Western blot of IE-3 isoforms in MCMV-injected eyes of immunosuppressed mice treated with either anti–IE-3 or control siRNA at various times p.i. A significant decrease in levels of the various IE-3 protein isoforms was noted. An eye from a single infected mouse was used for each of the lanes on the Western blot, which is representative of several such experiments performed on siRNA-treated mice. (**B1–B3**) Ratio of IE-3 isoform to β-tubulin (**$P < 0.001$, ***$P < 0.0001$). Statistical significance was calculated using a Student's *t*-test.

time points post infection and injected eyes were harvested for determination of virus titer by plaque assay. We observed that IE-3–specific siRNA produced a substantial decrease in MCMV titers over the time course of virus infection, compared with control siRNA (Fig. 3). We also observed a decrease in levels of the various IE-3 protein isoforms (Fig. 4). Interestingly, one of the protein isoforms produced by the *IE-3* gene appeared to be somewhat less affected by IE-3–specific siRNA. A 38-kDa, IE-3 antibody reactive protein, which appeared at a late stage of infection (day 14), showed levels comparable to those present in eyes treated with control siRNA. The nature and function of this protein relative to other IE-3 isoforms remains to be determined.

IE-3–Specific siRNA Alleviates MCMV Retinitis

An important component of retinal destruction following MCMV infection is the apoptotic death of uninfected cells that form part of a large "bystander" effect. Therefore, alleviation of the symptoms of MCMV infection requires that the death of uninfected cells be substantially reduced. To determine if inhibition of MCMV replication by IE-3 siRNA resulted in diminished bystander cell death, we performed TUNEL assays on frozen sections prepared from IE-3–specific and control siRNA-treated, MCMV-infected retinas. As can be seen in Figure 5, there is a significant decrease in numbers of TUNEL-staining cells in retinas of mice treated with IE-3–specific siRNA compared with retinas of mice treated with control siRNA. Hematoxylin and eosin staining indicated that the overall architecture of the retina is greatly improved in IE-3–specific siRNA-treated mice with an almost complete absence of full-thickness retinitis (Fig. 6A). Retinal folds are observed but usually in less than one-quarter of the retina (Fig. 6A). By using a previously published system of retinitis scoring,[12] we noted that the effect of siRNA on retinitis severity correlated with its effect on virus titers (Fig. 6B). This result demonstrates that

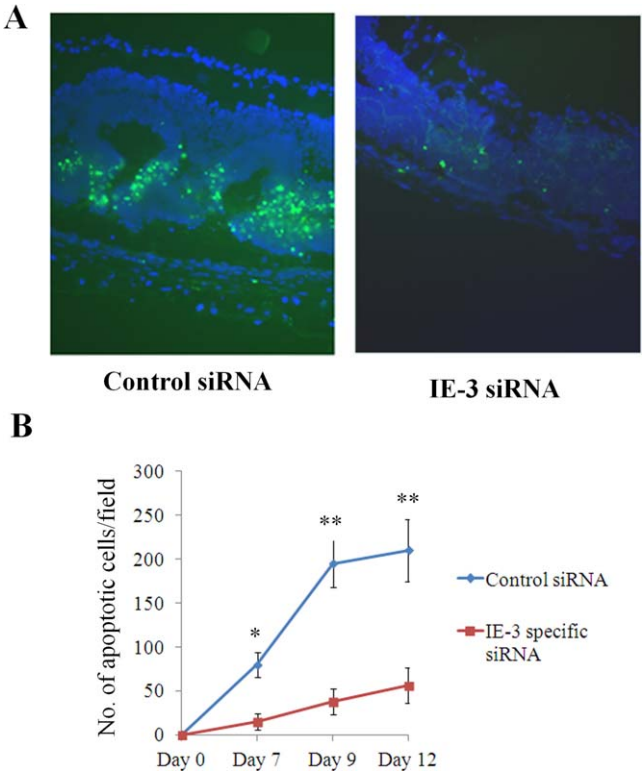

Figure 5. (**A**) Merged photomicrographs of staining of TUNEL (*green*) and DAPI (*blue*) in MCMV-injected eyes at day 9 p.i after treatment with either IE-3–specific or control siRNA. (**B**) Graph of the number of apoptotic cells in the injected eyes at various times post infection in MCMV-infected mice treated with either anti-IE-3 or control siRNA (*$P < 0.02$, **$P < 0.001$). Groups of three mice were used per experiment and statistical significance was calculated using a Student's *t*-test.

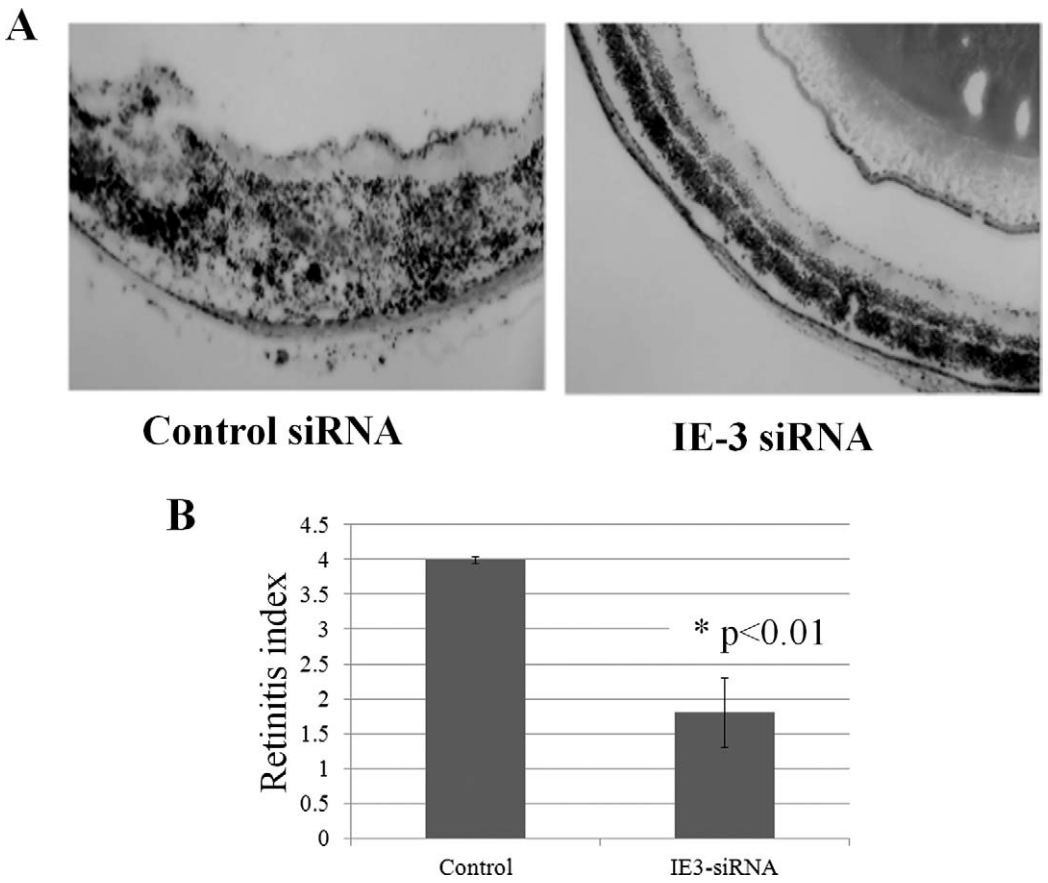

FIGURE 6. (**A**) Photomicrographs of H&E staining in MCMV-injected eyes at day 9 p.i after treatment with either IE-3–specific or control siRNA. (**B**) Graph of retinitis levels in the injected eyes in MCMV-infected mice treated with either anti–IE-3 or control siRNA at day 9 p.i. (*$P < 0.01$). Statistical significance was calculated using a Student's *t*-test.

inhibition of virus replication is beneficial to the survival of uninfected cells in the retina.

DISCUSSION

Small inhibitory RNAs are widely used to selectively inhibit gene expression both in vivo and in vitro. The process uses small RNAs to interfere with gene expression at both transcriptional and posttranscriptional levels by targeting newly transcribed RNAs for nucleolytic attack and also by causing transcriptional silencing of particular chromosomal regions, such as those rich in heterochromatin.[31–33] Not surprisingly, RNA interference (RNAi) has attracted considerable interest as a possible therapy for various types of virus infection. Numerous reports have documented the inhibitory effects of siRNA and micro RNA on both RNA and DNA virus infection in vivo and in vitro. These include RNA viruses, such as HIV-1[34–36]; hepatitis A, B, and C[37–39]; dengue virus[40]; influenza A virus[41]; and poliovirus,[42] and DNA viruses, such as herpes simplex virus type 1,[43] human papillomavirus,[44] Epstein-Barr virus,[45,46] and HCMV,[47,48] have also been reported to be susceptible to RNAi. Using siRNAs clinically, however, poses some challenges, including the development of adequate delivery methods and the elimination of "off-target" effects.

Murine CMV–induced retinitis is characterized by a large bystander effect, which results in destruction of uninfected cells. Prevention of this effect could lead to significant alleviation of the ocular symptoms of MCMV infection. We show here that siRNA directed against the IE-3 gene significantly reduces both virus titers and death of uninfected cells when administered intravenously. The accumulation of siRNA in the RPE layer of the retina is fortuitous because this appears to be the initial site of infection following MCMV inoculation. At the relatively low dose of siRNA administered to infected mice, no signs of toxicity or other abnormalities were observed, suggesting that this may be a viable means of treatment of retinitis. Because only a single layer of cells is targeted in the eye, rather than an entire tissue or organ, low levels of siRNA may be sufficient for ocular use of siRNA in vivo. Intravenous injection also is a minimally invasive means of administering siRNA and targeting the eye using this route may prove useful.

One question that is unanswered by the present study is the duration of siRNA effectiveness in vivo. In particular, we note that at day 14 p.i. there is a rise in virus titers compared with day 11, in IE-3–specific siRNA-treated samples. There also is no significant difference in virus titers between IE-3–siRNA treated and control siRNA-treated samples at this time point, in contrast to all other time points studied. Because only a single dose of siRNA was injected into the mouse 2 days before infection, it is possible that by this time, the anti–IE-3 siRNA has been degraded by nuclease action and is no longer present at a concentration sufficient to inhibit virus replication. Consistent with this possibility, we have noted that fluorescein-labeled siRNA ceases to be visible in the RPE layer approximately 10 to 12 days after injection (not shown). This would equate to approximately 8 to 10 days after virus infection, which fits with the increased virus titers observed at

day 14 p.i. We also have noted the appearance of a smaller IE-3 isoform of approximately 38 kDa, which appears at day 14 p.i. and is strongly expressed in eyes treated with IE-3–specific siRNA. The nature and function of this protein have yet to be determined, but it is possible that it could play a role in the increased virus titers observed at this time.

Current drugs, such as ganciclovir, valganciclovir, cidofovir, and foscarnet, have been effective against HCMV retinitis, but prolonged therapy with these drugs is associated with dose-limiting toxicities, thus limiting their utility.[9] Moreover, development of drug-resistant mutants has been observed.[9] Therefore, continuous efforts by researchers in the industry and academia are needed to develop newer candidates, such as siRNA targeting immediate early gene of cytomegalovirus, with enhanced antiviral efficacy and apparently minimal side effects.

Acknowledgments

Supported by National Institutes of Health Grant EY009169 and the Retinal Research Foundation.

Disclosure: **B. Marshall**, None; **J. Mo**, None; **J. Covar**, None; **S.S. Atherton**, None; **M. Zhang**, None

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