An Antiviral Small-Interfering RNA Simultaneously Effective against the Most Prevalent Enteroviruses Causing Acute Hemorrhagic Conjunctivitis

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Purpose. Acute hemorrhagic conjunctivitis (AHC), a highly contagious eye disease, is caused primarily by either enterovirus 70 (EV70) or coxsackievirus A24 (CVA24) infection. Yet methods to prevent or cure AHC are not available. Recent evidence has shown that small-interfering RNAs (siRNAs), mediators of posttranscriptional gene knockdown, can act as effective antiviral agents. Thus, the authors attempted to develop a novel siRNA-based anti-AHC agent effective against both EV70 and CVA24.

Methods. Concurrent screening of the entire viral genome sequences of EV70 and CVA24 using the CAPSID program identified five different siRNA candidates complementary to genome regions of both viruses. The antiviral potentials of these siRNAs were assessed by treating MRC5 and primary human conjunctival cells with the siRNAs and following this with viral challenge.

Results. Among the five siRNAs, AHCe-3D-3 siRNA showed excellent cytoprotective effects and dramatic decreases in virus replication and virus protein synthesis. This siRNA, targeting the virus polymerase 3D gene, also induced similar antiviral effects in primary human conjunctival cells.

Conclusions. These findings strongly suggest that the AHCe-3D-3 siRNA, homologous to two different AHC-associated enteroviruses, can provide equivalent antiviral activities against both AHC-causing enteroviruses. Such an siRNA may be developed as a clinically valuable AHC control agent. (*Invest Ophthalmol Vis Sci.* 2011;52:58–63) DOI:10.1167/iovs.09-5051

Acute hemorrhagic conjunctivitis (AHC), caused by a rapidly progressive virus infection, was first described in 1969.[1] Since that time, epidemic outbreaks of AHC have been reported in various countries, including Pakistan, Brazil, Singapore, Africa, Nepal, United States, Egypt, Cuba, China, and Korea.[2–10] Clinically, AHC is characterized by the rapid onset of painful conjunctival congestion, vascular dilatation, and edema.[4] AHC is highly contagious; thus, secondary attack rates in households with (initial) single patients are extremely high. However, neither preventive nor therapeutic treatment is available, though the symptoms may be addressed.

Serologic studies have indicated that enterovirus 70 (EV70) and coxsackievirus A24 (CVA24) are the major causative agents of AHC.[11,12] CVA24 and EV70 belong to distinct human enterovirus subgroups, human enterovirus C (HEC) and human enterovirus D (HED), respectively.[13] Each of these viruses has a positive single-stranded RNA genome coated with capsid proteins, including VP1 to VP4.[14] The genome sequences are very different. Enteroviral infection has been found to result in productive virus replication,[15–17] followed by irreversible damage to cells and tissues, that is directly linked to enterovirus-associated clinical complications.[18] Therefore, an antienteroviral agent that inhibited virus replication would be of great value.

RNA interference (RNAi) is a posttranscriptional gene-silencing phenomenon that causes degradation of target RNA in a sequence-specific manner.[19,20] Small-interfering RNAs (siRNAs), essential mediators of successful RNAi processes, are double-stranded, 19 to 23 nucleotides in size, and entirely complementary to target sequences. In the time since siRNA was first discovered, accumulating evidence has suggested that virus genome-specific siRNAs can serve as successful antiviral agents against a variety of viruses, including HIV, RSV, and HCV.[3,21] Indeed, we and other groups previously showed that several types of enterovirus infection, including that mediated by coxsackievirus B3 (CVB3), can be profoundly inhibited by treatment with virus-specific siRNAs, both in vitro and in vivo.[22–25] We recently reported that CVA24-specific siRNA had potent antiviral activity against CVA24.[26] To date, however, there have been no studies on siRNAs simultaneously effective against both enteroviruses causing AHC.

We therefore designed siRNAs homologous to both CVA24 and EV70 genome sequences to achieve anti-AHC effects, caused by these two agents. The therapeutic potencies of such siRNAs were examined in primary human conjunctival cells and in cells permissive for viral replication. We report here the successful design of siRNAs effective against both enteroviruses that cause AHC.

Materials and Methods

Cell Culture

MRC5 and HeLa cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Human conjunctival fibroblast cells were isolated from human tissues of different donors and cultured as described.[26] The study protocol was reviewed and approved by the Institutional Review Board of the Asan Medical Center (Seoul, Korea), and all patients provided written informed consent. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum, L-glutamine

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(2 mM), and penicillin (100 IU/mL)/streptomycin (50 μg/mL) at 37°C in a 5% (vol/vol) CO_2 incubator.

Virus Manipulation

EV70 (EV70-J670/71; #VR836) was purchased from the ATCC. CVA24 strains, originally isolated from clinical samples in Korea, were generous gifts of the Korea Center for Disease Control and the Korea Research Institute of Chemistry Technology. Viruses were propagated in either HeLa (CVA24) or MRC5 (EV70) cells and were titered using the $TCID_{50}$ criterion. For quantification of progeny virus production, cells were infected at a multiplicity of infection (MOI) of 5 for 1 hour with or without siRNA pretreatment. After virus inocula were washed out, cells were cultured in fresh medium. Media and cells were harvested, and a $TCID_{50}$ assay was performed for quantification of each assay.

siRNA Design and Treatment of Cells

Using the CAPSID program (Convenient Application Program for siRNA Design), we identified 21-nt duplex siRNAs with dTdT 3′-overhangs based on the genome sequences of CVA24 (GenBank accession no. D90457) and EV70 (GenBank accession no. D00820). Synthetic siRNAs were manufactured by Dharmacon (Lafayette, CL), with a "ready-to use" option. Control siRNAs tagged with the red fluorescent dye Cy-3 and modified siRNAs containing single phosphates at the 5′ position were purchased from Dharmacon. Cells were transfected with 100 nM siRNA complexed with transfection reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA) in multipurpose medium (OPTI-MEM; Invitrogen). After 4 hours, growth medium containing 10% (vol/vol) serum was added without removal of the transfection mixture. Cells were incubated for an additional 8 hours and were infected with viruses at an MOI of 5.

Immunocytochemistry and Hoechst 33342 Staining

Immunocytochemistry was performed as described.[26] Briefly, cells were fixed with 4% (vol/vol) paraformaldehyde, permeabilized with 0.05% (vol/vol) Triton X-100 in 0.1 M phosphate buffer (pH 7.4), and incubated with primary antibody specific for virus VP1 protein (CVA24; K-MAB898 and EV70; MAB843; Chemicon, Billerica, MA). Immunopositive signals were visualized using FITC-conjugated secondary antibody. For Hoechst 33342 staining, cells were fixed with absolute methanol for 30 minutes at room temperature and were washed once with PBS. Nuclear morphology of cells was visualized after incubation with 1 μg/mL Hoechst 33342 dye (Invitrogen) for 30 minutes at 37°C. Cells were examined by fluorescence microscopy with a UV filter to determine morphologic changes in nuclei.

Western Blot Analysis

Cells infected with CVA24 or EV70 in the presence or absence of siRNAs at a six-well scale were harvested 10 to 12 hours after infection and were lysed in 100 μL lysis buffer. The lysates were boiled at 100°C with 6× sample buffer, and the denatured proteins were separated on 10% SDS-PAGE and were analyzed by immunoblotting. The membrane was blocked for 1 hour in TBS/0.1% Tween 20 containing 5% dry milk and was incubated subsequently in the presence of the primary VP1 antibody overnight at 4°C. After the membrane was incubated in the peroxidase-conjugated mouse second antibody for 1 h at room temperature, it was developed using enhanced chemiluminescence reagents from Pierce (Rockford, IL). The antibody against β-actin (Sigma, St. Louis, MO) was used as a control for protein input.

Statistical Analysis

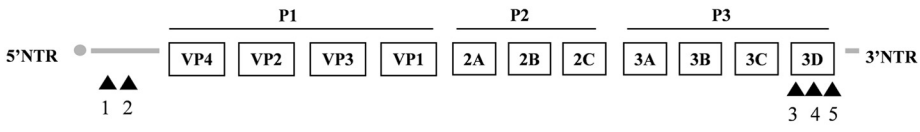
Data were explored by univariate analysis of variance (ANOVA) making use of the SPSS (Chicago, IL) program and were next compared using the paired *t*-test or descriptive statistics. Statistical significance was defined as $P < 0.05$.

Results

Selection of siRNA Candidates for AHC-Associated Enteroviruses and Validation of Antiviral Effects in MRC5 Cells

Using the CAPSID program, which identifies potential siRNA by systematically inspecting conserved sequence patterns from multiple inputs (http://cms.ulsan.ac.kr/capsid), we selected five siRNA candidates after screening the two entire virus genomes, both approximately 7.3 kb in length (Fig. 1). Two of these siRNAs theoretically bound to the 5′-nontranslational regions, and the other three bound to the 3D virus polymerase-encoding genes (Fig. 1a). All siRNA nucleotide sequences and the exact target positions in virus genomes are described in Figure 1b. To assess the antiviral activities of the siRNAs, MRC5 cells were preincubated with each siRNA and subsequently challenged with either CVA24 or EV70. MRC5 cells efficiently supported the replication of both viruses, leading to irreversible cytotoxicity, characterized by initial cell rounding, nuclear condensation (by arrows), and eventual cell lysis, within 15 to 20 hours after infection (Fig. 2). In contrast, the cells treated with AHCe-3D-3 showed dramatic anticytopathic effects against both EV70 and CVA24 infection. We also found that this siRNA sharply reduced virus protein (VP1) synthesis, as indicated by the decrease in VP1-positive cells (immunocytochemistry)[27] and the decrease of VP1 expression (Western blot analysis). Significant reduction in progeny virus production also was consistently observed in the cells treated with AHCe-3D-3, not control siRNA (Fig 3). However, we did not show the profound antiviral effects by other siRNAs except AHC3–3D-3. Therefore, we selected AHCe-3D-3 siRNA targeting the 3D-

(a)



(b)

	Name	Position	Sequence (5'-3')
1	AHCe-5'-1	5'NTR (CVA24: 582, EV70 : 578)	AUGGCUGCUUAUGGUGACA
2	AHCe-5'-2	5'NTR (CVA24: 584, EV70 : 580)	GGCUGCUUAUGGUGACAAU
3	AHCe-3D-1	3D (CVA24: 6698, EV70: 6614)	GCAUUUGACUACACAGGUU
4	AHCe-3D-2	3D (CVA24: 6703, EV70: 6619)	UGACUACACAGGUUAUGAU
5	AHCe-3D-3	3D (CVA24: 6985, EV70: 6903)	UGGUGAUGAUGUAAUUGCU

Figure 1. Genome organization and siRNA sequence information. (**a**) A virus genome consisting of a single-stranded RNA with a 5′-UTR; P1, P2, and P3 genes; and a 3′-UTR. P1 encodes four structural proteins (VP1–4), and P2 and P3 encode seven nonstructural proteins (2A–C, 3A–D). (**b**) Table showing the siRNA nucleotide sequences and target locations in the CVA24 and EV70 genomes.

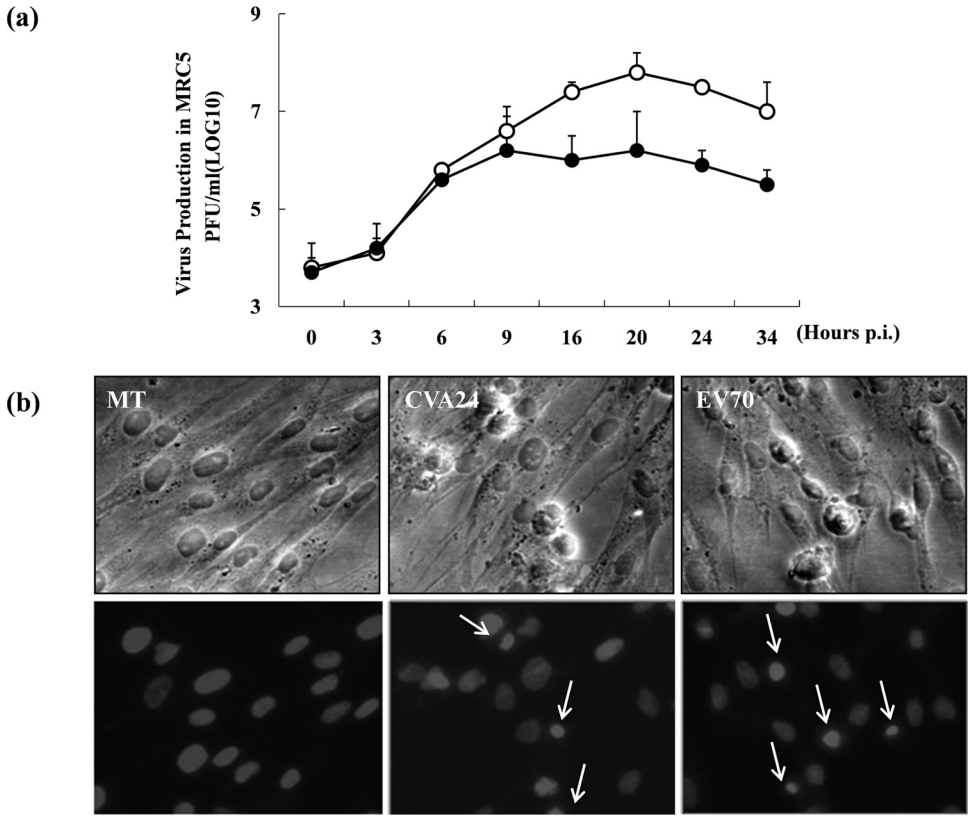


FIGURE 2. Characteristics of MRC5 cells after virus infection. Cells were infected with CVA24 (○) or EV70 (●) at an MOI of 5. (**a**) Progeny virus production at 3, 6, 9, 16, 20, 24, and 34 hours after infection was measured by the $TCID_{50}$ assay ($n = 3$). (**b**) Cytopathic effects were assessed by light microscopy/fluorescence microscopy (LM/FM) after Hoechst 33342 staining at 16 hours after infection. *Arrows*: nuclear condensations.

conserved region of both EV70 and CVA24. These findings indicated that AHCe-3D-3, targeting the 3D-conserved region of AHC-causing enteroviruses, directly interfered with virus replication.

Protective Effects of AHCe-3D-3 in Primary Human Conjunctival Cells

To verify the therapeutic potential of AHCe-3D-3 siRNA, we assessed its effect in primary cultures of human conjunctival cells. As expected, both CVA24 and EV70 replicated well in such cells, resulting in a pattern of irreversible cytopathic effects similar to that observed in permissive MRC5 cells (data not shown). Treatment with AHC3e-3D-3, but not control siRNA, significantly inhibited both virus replication and cytotoxic effects in human conjunctival cells (Fig. 4). These protective effects were consistent, regardless of virus type or the origin of the primary cells. These findings provide further evidence that AHCe-siRNA protects natural human host cells from virus-induced cytotoxicity by direct interference with virus replication.

To determine whether AHCe-3D-3 could induce antiviral activities in clinical situations, we examined its antiviral potency against several clinically isolated wild-type AHC-enteroviruses. We found that AHCe-3D-3 exerted protective effects against all these isolates (data not shown). When we assessed the target sequences in AHC-associated wild-type enteroviruses, we found that these sequences were completely conserved in all the viruses tested (data not shown).

One of the major criteria for successful siRNA activity is selection of an effective target site, which can depend heavily on structural features of the siRNA target region. The secondary structures of genome regions targeted by the siRNA candidates were analyzed using Mfold software.[28] The sequence targeted by AHCe-3D-3 was easily accessible to siRNA, as indicated by the secondary loop structure in the region of the recognition site (Fig. 5). In contrast, the AHCe-siRNAs that exhibited no antiviral activities were complementary to tightly ordered structures with many hydrogen bonds.

DISCUSSION

Given that AHC is caused by two distinct enteroviruses, EV70 and CVA24, any therapeutic siRNA should be equally effective against both viruses. We recently developed software (CAPSID) to design siRNAs targeting well-conserved regions within several different viruses.[29] The results presented here provide clear evidence that an siRNA (AHCe-3D-3) designed using the CAPSID program exhibited similar levels of potent antiviral activity against both CVA24 and EV70 by targeting a sequence homologous in the two viruses. The protective effects of the siRNA in permissive MRC5 cells were retained when human conjunctival cells, the target tissue of AHC-associated enteroviruses, were tested.

Although five possible siRNA candidates were selected by application of CAPSID, only one, AHCe-3D-3, showed substantial antiviral effects. For preferential degradation of target RNA after hybridization with an siRNA, the 3′ end region of the target should be sterically recognized by the 5′ seed region in the guide strand of the siRNA.[30,31] An open loop structure in the target RNA, thus freely exposing unpaired nucleotides, is preferred, whereas a highly ordered stem structure restricts target accessibility by siRNA. Secondary structure analysis predicted that the target region of AHCe-3D-3, but not those of the other siRNA candidates, would have a considerable component of hairpin structure. Additionally, the siRNA initial recognition region was thermodynamically unstable, allowing the target RNA to form a complex with an siRNA-induced silencing complex (RISC), which mediates RNAi.[32]

Active degradation of the target RNA in the RNAi approach requires the siRNA guide strand to be specifically present in the RISC. Recent studies on the three-dimensional conformation of

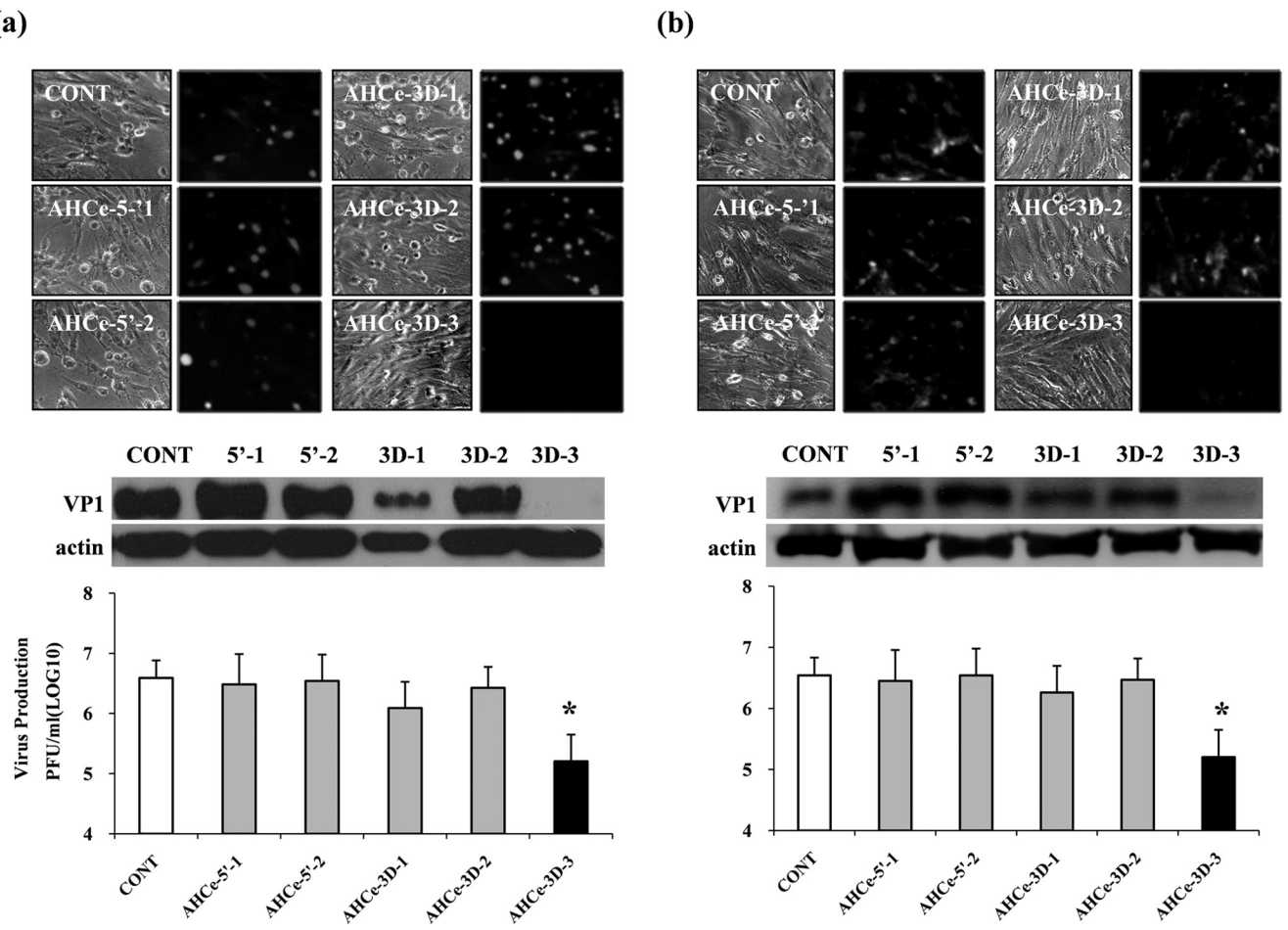


FIGURE 3. Antiviral efficacy of siRNAs complementary to AHC-associated enteroviruses in MRC5 cells. Cells were preincubated after infection with each siRNA for 12 hours and next were inoculated with either CVA24 (**a**) or EV70 (**b**) at an MOI of 5 for 15 hours or 12 hours, respectively. Loss of morphologic integrity was assessed by light microscopy, and virus protein expression was determined by immunocytochemistry and Western blot analysis. Progeny virus production was determined by $TCID_{50}$ ($n = 3$). AHCe-3D-3 siRNA was effective against both EV70 and CVA24. *Open bars*: infection in the presence of control siRNA; *filled bars*: infection in the presence of various siRNAs. CONT, control siRNA. $^*P < 0.05$.

the RISC particle showed that the 5′ terminal of the siRNA was incorporated into the MID domain of the Agonaute (Ago) protein, an essential component of RISC.[33,34] This incorporation is absolutely required if the residual portion of the siRNA strand is to be properly positioned within the Ago pocket in a sequence-independent manner. Moreover, the 5′-phosphate group of siRNA was absolutely required for such productive assembly. This configuration, in turn, was found to determine the exact site for the proteolytic activity of RISC, further supporting the functional importance of the 5′-phosphate.[33,35] Because conventionally available synthetic siRNAs are in the 5′-OH form and not the 5′-phosphate (5′-P) form, we prepared enterovirus-specific siRNAs containing the required phosphates and evaluated the antiviral activities of these materials. Unexpectedly, there was no significant difference between the two forms either in inhibition of virus replication or in attenuation of cytotoxicity (data not shown). In the cytosol, the 5′-OH form may be converted to the 5′-P form by cellular kinases in the presence of adenosine triphosphate.[19] As a result, the antiviral activity of the 5′-OH form of synthetic siRNA can be as effective as that of the 5′-P form, indicating that the in vitro 5′-phosphate modification is not required for the effectiveness of an siRNA when used as a therapeutic agent.

The siRNA with AHCe-attenuating potential identified in this study had a sequence complementary to the gene encoding the 3D RNA-dependent RNA polymerase, which has a highly conserved sequence.[14] Because of the lack of proofreading ability of various virus RNA polymerases, even supposedly single species of particular RNA viruses really consist of collections of quasi-species, with genomic diversity and genetic instability, in nature.[36,37] Targeting of highly conserved regions is thus essential for the development of siRNA-based therapeutic agents inhibiting the replication of RNA viruses. For example, an siRNA-targeting HEB, including CVB3, could be developed after study of the 3D region.[11,22] Genes encoding other nonstructural proteins, such as 2A and 2C, have been identified as promising alternatives for RNA targeting.[24,38] The *cis*-acting element (CRE) region within 2C is a particularly promising target for the design of potent anti-HEB siRNAs, inhibiting replication of viruses within the enterovirus B subgroup.[38] The CRE region, a core *cis*-acting element essential for virus genome amplification, is extremely sensitive to genetic alteration.[39] In addition, the CRE region spatially forms a large loop, allowing easy access to the virus replication machinery and thus to siRNA.[38,39] Indeed, CVA24-specific siRNA targeting the CRE region showed high-level anti-CVA24 activity against clinically isolated CVA24s of diverse sequences.[26] Nevertheless, an siRNA targeting the CRE region was not selected in this work, in which we targeted CVA24 and EV70 viruses, which belong to different enterovirus subgroups (HEC and HED). Close examination of sequences in the CRE region revealed multiple mismatches between HEC and HED. Two sequence mis-

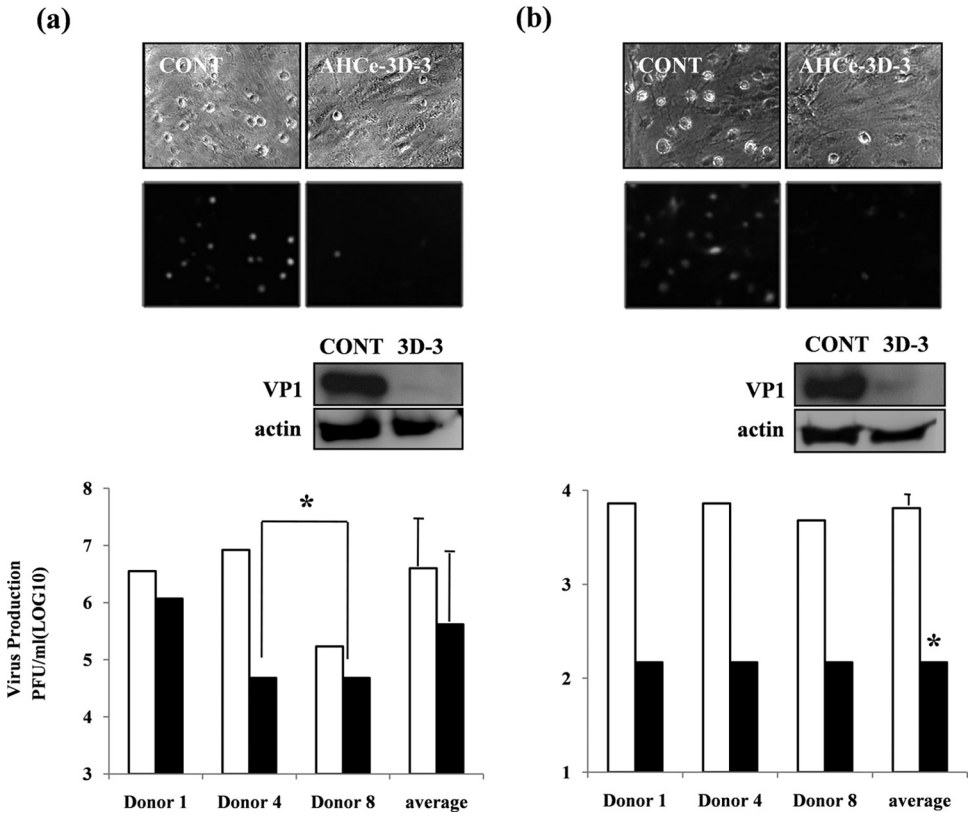


FIGURE 4. Protective effects of AHCe-3D-3 in primary human conjunctival cells after infection. Human conjunctival cells were treated and analyzed as described in the legend to Figure 3. AHCe-3D-3 treatment actively protected infected conjunctival cells from virus-induced damage by CVA24 (**a**) and EV70 (**b**). *Open bars*: infection in the presence of control siRNA; *filled bars*: infection in the presence of AHCe-3D-3. $^*P < 0.05$.

matches were located at positions 10 and 11 from the 5′ end of CAPSID-suggested siRNA sequences. Because actual cleavage of target RNA occurs between these positions, absolute sequence identity of target RNA and siRNA is required.[33] Recombination among enterovirus genomes is a relatively frequent phenomenon, primarily at the noncoding region of the EV genomes, including 2C and the highly conserved 3D region. Therefore, there is still a chance that some specific siRNAs fail to inhibit the viral replication.[38]

For siRNA-based agents to achieve therapeutic benefits in clinical settings, a safe and effective siRNA delivery system is needed.[40] Thus, it is practical to develop siRNA-based agents for human diseases that are amenable to topical treatment. Possible target tissues or organs for local treatment include the skin, eyes, and mucosal layers. Indeed, practical applications of siRNAs in the treatment of eye diseases have been reported. For example, SIRNA-027 was found to be successful in the treatment of age-related macular degeneration.[41,42] In addition,

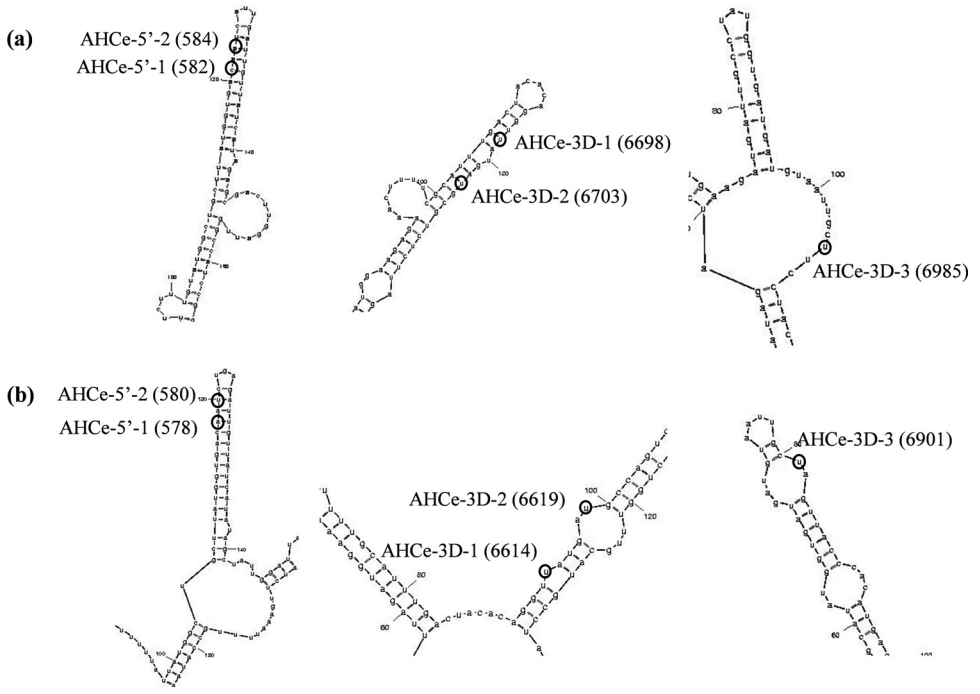


FIGURE 5. Predicted structure of siRNA target regions. The secondary structures of genome regions (**a**, CVA24; **b**, EV70) targeted by the siRNA candidates were analyzed using Mfold software (http://frontend.bioinfo.rpi.edu/applications/mfold). *Circles*: target positions recognized by the first seed sequences of the designated siRNAs; *lines*: hydrogen bonds.

direct administration of anti-RSV siRNA to the respiratory tract showed therapeutic efficacy against RSV infection.[43]

In conclusion, our results strongly suggest that an siRNA homologous to the two different enteroviruses that induce AHC has potent antiviral activity against these AHC-related enteroviruses. Use of this siRNA may therefore be clinically feasible in the treatment of patients with AHC.

Acknowledgments

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