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Original Article

Small interfering RNA targeting for infected-cell polypeptide 4 inhibits herpes simplex virus type 1 replication in retinal pigment epithelial cells

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Abstract

Background: This study sought to inhibit herpes simplex virus type 1 replication using small interfering RNA which targeting infected-cell polypeptide 4 genes to mediate transcription of early and late viral genes in herpes simplex virus type 1 lytic (productive) infection in retina epithelial cells.

Methods: After pre- or post-infecting with herpes simplex virus type 1, small interfering RNAs were transfected into retina epithelial cells. The antiviral effects of small interfering RNA were evaluated by Western blot, plaque assays, indirect immunofluorescence and reverse transcription polymerase chain reaction. The viral titre was detected by the 50% tissue culture infective dose method.

Results: Small interfering RNA decreased infected-cell polypeptide 4 expression in retina epithelial cells that were infected with herpes simplex virus type 1 before or after small interfering RNA transfection. Compared with herpes simplex virus type 1 infection alone or transfection with negative control small interfering RNA, the viral titre and the retina epithelial cell cytopathic effect were significantly decreased in retina epithelial cells transfected with infected-cell polypeptide 4-targeting small interfering RNA (50 and 100 nM) ($P < 0.05$). The small interfering RNA effectively silenced herpes simplex virus type 1 infected-cell polypeptide 4 expression on both mRNA and the protein levels ($P < 0.05$). The inhibition of infected-cell polypeptide 4-targeting small interfering RNA on infected-cell polypeptide 4 protein expression was also verified by Western blot in herpes simplex virus type 1 infected human cornea epithelial cell, human trabecular meshwork cells and Vero cells.

Conclusions: Infected-cell polypeptide 4-targeting small interfering RNA can inhibit herpes simplex virus type 1 replication in retina epithelial cells, providing a foundation for development of RNA interference as an antiviral therapy.

Key words: HSV-1, siRNA, immediate early infected-cell polypeptide 4, retinal pigment epithelium.

Introduction

Herpes simplex virus type 1 (HSV-1) is a double-stranded DNA virus with 152-kb genome that infects the majority of the human population. It can express two distinct transcriptional patterns: lytic (productive) infection and latency infection. During lytic infection, approximately 74 genes encoded within the viral genome are classified as immediate-early (IE or α), early (β) and late (γ) genes by RNA polymerase II.[1,2]

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The immediate-early gene infected-cell polypeptide 4 (ICP4) is a major regulatory gene required for efficient transcription of early and late viral genes that make it essential for lytic infection.[1] ICP4 protein, which is synthesized immediately after the infection, binds DNA in a sequence-specific manner as a homodimer.[3,4] Su *et al.* reported that ICP4 protein plays an important role in mediating the circularization of the viral genome as ICP4 deletion mutant d120 failed to assume an endless or circular conformation following infection.[5] In addition, ICP4 has been previously associated with the formation of viral replication compartments and increased formation of transcription preinitiation complexes in a TATA box-dependent manner.[6–8] Studies have suggested that ICP4 intervenes at a very early step in the formation of the transcription initiation complex and that its mechanism of recruitment is fundamentally different from that of other activators, such as VP16.[6] The VP16 gene is a late gene whose expression is dependent on IE, early-protein functions and viral DNA replication.[9,10] The activation domain of VP16 has been suggested to recruit general transcription factors and co-activators to IE promoters via protein–protein interaction and to reduce the association of histones with those promoters; thus, VP16 activates transcription of the IE genes and results in a cascade of gene transcription considered to be essential for HSV-1 replication.[9,11] Both VP16, which activates transcription of the five IE genes, and ICP4, which activates the remainder of the HSV genome, function at the level of transcription initiation, contributing to the lytic cascade of viral gene expression.

Retinal pigment epithelial cells (RPEs), which form the outermost layer of the retina, play a vital role in the maintenance of the human retina and provide a target for viral invasion. Evidence have shown that HSV-1 can infect RPEs *in vitro* and *in vivo*.[12–14] HSV-1-based amplicon vectors can rapidly and efficiently transfer genes into RPEs *in vivo*.[15] Retinitis, especially acute retinal necrosis (ARN), which is caused by HSV-1 infection, always leads to severe complications in patients.[16,17] ARN is a blinding disease marked by rapidly progressive peripheral retinal necrosis that was first described in 1971. HSV-1 is the second most common cause of ARN,[16] and the leading cause in ARN patients older than 25 years.[18] ARN may result from the direct invasion of HSV-1 or a recurrence of a previous episode of retinitis, keratitis and/or encephalitis caused by the virus.[19,20] Furthermore, latent HSV-1 infections, which occur in more than 90% of adults, may be triggered by such events as trauma, UV irradiation, neurosurgery or high-dose corticosteroids.[21] Therefore, suppressing productive infection of the virus is vital in clinical practice to control HSV-1-associated diseases.

RNA interference (RNAi) is a post-transcriptional process by which a specific mRNA is targeted for degradation as a means of inhibiting the synthesis of the encoded protein.[22] RNAi technology has been reported to be an ideal tool for treatment of infectious diseases by silencing viral genes to inhibit replication.[23,24] RNAi has also been reported to inhibit HSV-1 replication by using small interfering RNAs (siRNAs) targeting ICP6,[25] VP16[26] and glycoprotein E.[27] It has been reported that ICP4 mRNA is the target of HSV-1 miR-H6,[28] and its expression can also be inhibited by LAT sRNA2.[29] In this study, siRNA was used to inhibit HSV-1 replication in RPEs by silencing viral ICP4 gene. We showed that siRNA significantly decreased the expression of ICP4 and inhibited viral replication in RPEs. In addition, we found that ICP4-targeting siRNA (ICP4-siRNA) can still inhibit viral replication post-infection by HSV-1, which mimics the clinical treatment after the HSV-1 lytic infection. Moreover, ICP4-siRNA also downregulated ICP4 protein expression in human corneal epithelial cells (HCEs), human trabecular meshwork cells (HTMs) and Vero cells, which further supported that ICP4-siRNA inhibited HSV-1 replication *in vitro*.

Methods

Cell and virus

The retinal pigment epithelium (cell line D407) was cultured in DMEM/F12 supplemented with 10% newborn bovine serum (Gibco, Grand Island, NY USA) at 37°C in a 5% CO_2-95% air incubator. Stocks of the HSV-1 (F strain) were propagated on Hep-2 cells, which were grown in DMEM/F12 with 10% newborn bovine serum. African green monkey kidney cells (Vero cells) were grown in 1640 supplemented with 10% newborn bovine serum. The HCE cell line[30] was cultured in DMEM/high glucose supplemented with 10% foetal bovine serum (Gibco), 10 ng/mL human epidermal growth factor (EGF; Sigma, St. Louis, MO, USA), 5 μg/mL human transferrin (Sigma), 5 μg/mL insulin and 0.4 μg/mL hydrocortisone (Gibco BRL). An HTM cell line[31] was cultured in DMEM/F12, containing 10% foetal bovine serum. These cells were infected with HSV-1 using a previously described method.[14,32] The viral titre was determined according to a previously described method.[14,32] Briefly, cells infected by HSV-1 were harvested at the indicated time, frozen and thawed repeatedly, and then centrifuged at 3000 rpm for 30 min to remove cellular debris. The supernatant with aliquots of serial dilutions was used to infect Vero cells in triplicate.

siRNA generation and transfection

Three siRNAs against ICP4 and a negative control siRNA (mock) were chemically synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The mock does not target any known gene. siRNA pools were reconstituted by rehydration in enzyme-free water (RiboBio, Guangzhou, China), divided to a final concentration of 20 μM per tube and then stored at −80°C until used.

RPEs were transfected with 50 nM siRNAs or mock followed by infected with HSV-1 (MOI 0.1) 24 h post-transfection. By using Western blot analysis, we found that one among three siRNAs revealed more effective inhibition on ICP4 expression than the other two (data not shown). So it was used in the subsequent experiments. The sequences of the siRNA duplexes were 5′ GCAACAGCAGCUCCUU CAUdTdT 3′ and 3′ dTdTCGUUGUCGUCGAGGA AGUA 5′, and the target sequences in ICP4 of siRNA were 5′-GCAACAGCAGCTCCTTCAT-3′.

The siRNA transfections were performed pre- or post-viral infection. For experiments in which the cells would be pretreated with siRNA, RPEs were grown to 70–80% confluence and then transfected with specific (50 and 100 nM, +siRNA) and mock siRNAs (mock) or without siRNA (–siRNA) by Lipofectamine 2000, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Cells were incubated in the transfection mixture for 4 h and further cultured in DMEM/F12 with 10% newborn bovine serum. After 24 h, the cells were infected with MOI 0.1 of HSV-1 for 1 h, with gentle 15-s shaking every 15 min to allow viral absorption. Then cells were further cultured with serum-free DMEM/F12 at 37°C, 5% CO2. For experiments in which the cells would be treated with siRNA following virus infection, RPEs were grown 90% confluence. Then cells were infected with MOI 0.1 of HSV-1, followed by transfection with siRNAs at 2, 6, 12 h p.i. By using Western blot analysis, we found that the dramatic inhibition occurred in cells transfected with siRNA at 2 h p.i. (data not shown). So transfection at 2 h p.i. was applied to conduct the present study. At the indicated time, cells were harvested for further analyses.

Plaque test

Plaque assay was conducted as described in previous literature.[27] For experiments in which the cells would be pretreated with siRNA, RPEs were grown in 12-well plates to 70–80% confluence and then transfected with siRNA and mock siRNA by Lipofectin 2000. 24 h post-transfection, the cells were infected with MOI 0.1 of HSV-1 for 1 h. Then cells were overlaid with 1 mL of a 1:1 mixture of low melting-temperature agarose (NuSieve GTG Agarose, Cambrex Bio Sci, Inc. Charles City, IA, USA) and 2 × DMEM/F12. For experiments in which the cells would be treated with siRNA following virus infection, RPEs were grown in to 90% confluence, followed by infection with MOI 0.1 of HSV-1 for 2 h, then transfected with siRNAs. At 4 h post-transfection, the cells were overlaid with a mixture of low-melting-temperature agarose and DMEM/F12. After incubation for 48 h, the agarose was removed and plates were stained with crystal violet for 20 min and then photographed. Plaque sizes were measured by Photoshop software and calculated by ImageJ software (http://rsbweb.nih.gov/ij/index.html). Experiments were repeated at least three times.

Immunofluorscence microscopy

RPEs grown on a glass coverslip in 12-well chamber dishes were used for indirect immunofluorescence analysis according to the method described previously.[32] Slide-mounted cells were infected with HSV-1 and transfected with siRNA as described earlier. At the indicated time, slides were incubated with mouse anti-human monoclonal antibody that recognizes HSV-1 ICP4 immediate early protein (Abcam, Cambridge, UK) at 4°C overnight. After being rinsed several times with phosphate-buffered saline (PBS), cells were incubated with FITC-conjugated secondary goat anti-mouse IgG antibody (Zhongshan Goldenbridge, Beijing, China) at 37°C. After 1 h, the slides were rinsed with PBS, the nuclei were stained with propidium iodide, and they were sealed with Antifade Solution (Applygen, Beijing, China). Cells were then observed using a confocal laser scanning microscope (Carl Zeiss, Jena, Germany). In addition, cells incubated with PBS (instead of the first antibody) were used as negative controls. Each experiment was repeated in triplicate.

Western blot analysis

The cell samples were frozen and thawed three times and then centrifuged at 12 000 rpm for 30 min at 4°C to remove cellular debris. Protein content in the supernatant was determined by the bicinchoninic acid method, using BSA as the standard. Western blot was conducted according to an established method.[33] Briefly, cells' lysates were boiled in a sample buffer for 5 min. Protein was loaded in each lane of 8% sodium dodecylsulfate-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes for electrophoresis, and blocked in TBST (5% fat-free dry milk, 0.1% Tween 20, 150 mM NaCl, and 50 mM Tris at pH 7.5) for 2 h. The membranes were exposed to 1 μg/mL of mouse anti-ICP4

antibodies (Abcam) or 0.2 μg/mL mouse anti-GAPDH (KangChen, Shanghai, China) separately and incubated overnight at 4°C. They were then incubated with a secondary goat anti-mouse IgG antibody (Zhongshan Goldenbridge, Beijing, China) for 1 h. Protein bands were visualized with the use of a kit of chemiluminescence Phototope (R)-HRP Western Blot detection system (Cell Signaling Technology, Inc., Danvers, MA, USA) and exposed by Kodak Imaging Station 4000MM (Kodak, Rochester, NY, USA). These average data were obtained from at least three independent experiments.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Cellular total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Subsequently, RNA was reverse-transcribed into cDNA by a RevertAid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA). Then cDNA was amplified by a kit of GoTaq Green Master mix (Promega, Madison, WI, USA).The VP16 primers were forward: 5′-GGTCGCAACAGAGGCA GTCA-3′ and reverse: 5′-CCCGAACGCACCCAAA TC-3′(418 bp), whereas the GAPDH primers were forward: 5′-GCACCGTCAAGGCTGAGAAC-3′ and reverse: 5′-TGGTGAAGACGCCAGTGGA-3′ (138 bp). PCR conditions were 94°C for 5 min and (94°C for 30 s, 55°C for 30 s and 72°C for 30 s for 25 cycles) 72°C for 5 min. PCR bands in agarose gels were analyzed with Kodak Molecular Imaging software. These experiments were repeated at least three times to get average data. Relative VP16 RNA levels were normalized to GAPDH RNA levels.

Statistics analysis

All experiments were conducted a minimum of three times with similar results, and quantitative data are expressed as means ± standard deviation. To compare the differences of viral titre and expression of mRNA (RT-PCR) or protein (Western blot) among the different treated cells, statistical analysis of data was performed by one-way ANOVA (SPSS 17.0, SPSS, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

Comparison of inhibitory effect of siRNAs pre- or post-HSV-1 infection

RPEs were transfected with siRNA (50 nM) pre- or post-HSV-1 infection Multiplicity of Infection (MOI 0.1). The inhibitory effects of siRNA were shown in Figure 1, First, the transfection of siRNA was administered 24 h prior to infection. At 24 h post-infection (p.i.), siRNA had inhibited the expression of ICP4 protein, whereas there were no effects on the expression for mock siRNA (Fig. 1a). Plaque test demonstrated that not treating with siRNA resulted in formation of a larger number of plaques, which result was similar to the mock siRNA. However, treatment with ICP4-siRNA significantly decreased the plaque sizes and numbers (Fig. 1c,e, $P < 0.05$). Second, we infected RPEs with HSV-1 for 2 h before transfecting with 50 nM ICP4-siRNA. The results showed that siRNA also inhibited ICP4 expression compared with the mock siRNA and not treating with siRNA (Fig. 1b). There was no expression of ICP4 in control cells, which were not infected with HSV-1, and plaque assays also showed that siRNA dramatically decreased the number and the size of plaques (Fig. 1d,f, $P < 0.05$), similar to those transfected prior to infection.

ICP4-siRNA reduces HSV-1 levels in RPEs

RPEs were infected with HSV-1 of MOI 0.1 for 2 h, followed by transfecting siRNAs into the cells. At 12 h p.i., the viral titre in cells transfected with ICP4-siRNA was much lower than that in the mock siRNA and in cells not treated with siRNA (Fig. 2a, $P < 0.05$). At 24 h p.i., the viral titre was greatly increased compared with that of 12 h p.i. However, the pattern of virus titre among different treated cells was inconsistent with those at 12 h p.i. In general, ICP4-siRNA reduced viral replication by more than 69% at 12 h p.i and more than 95% at 24 h p.i. compared with its propagation in the mock siRNA-treated cells (Fig. 2b, $P < 0.05$). However, although the virus titre tends to have lower expression in 100 nM siRNA-treated cells than in 50 nM siRNA-treated cells, the statistical analysis showed no significant differences ($P > 0.05$).

ICP4-siRNA inhibits the cytopathic effect of RPEs infected with HSV-1

After HSV-1 infection of MOI 0.1 2 h, RPEs were transfected with ICP4-siRNA, and cell morphological changes were observed under a phase-contrast microscope (Fig. 3). Following HSV-1 infection and up to 6 h p.i., there were no obvious differences in cellular morphology among cells transfected with ICP4-siRNA (50 and 100 nM), the mock siRNA, without siRNA (0 nM) and the uninfected RPEs. At 12 h p.i., the cytopathic effect (CPE) could be observed in the mock siRNA and siRNA-untreated control cells, but not in the cells transfected with ICP4-siRNA. Infected cells are usually displayed in clusters, and many individual cells remained uninfected. At 24 h p.i., CPE increased dramatically,

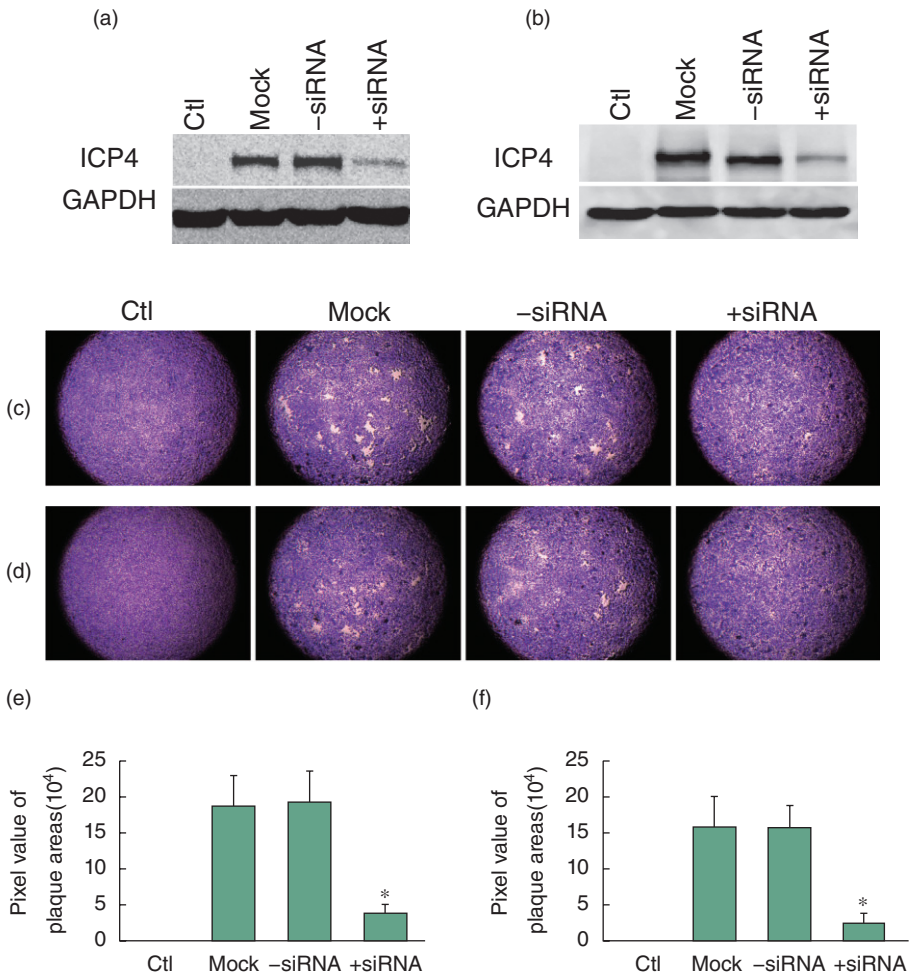

Figure 1. Comparing the inhibitory effects of small interfering RNA (siRNA) on retinal epithelial cells (RPEs) infected with herpes simplex virus type 1 (HSV-1) by Western blot analysis (a, b) and by plaque assays (c–f). (a, c, e) RPEs were transfected with siRNAs 24 h prior to infection with HSV-1. At 24 h post-infection (p.i.), cells were harvested. (b, d, f) RPEs were infected with MOI 0.1 of HSV-1 for 2 h followed by transfection with siRNAs. At 24 h p.i., experiments ended. (e, f) The average plaque areas of (c) and (d) separately. Cells were treated with siRNA (+siRNA or mock RNA) or without siRNA (–siRNA). The control group (Ctl) consisted of cells without virus infection and siRNA treatment. Each data is the mean value of three independent assays. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICP4, infected-cell polypeptide 4.

and many giant multinucleated cells could be seen in the mock siRNA and siRNA-untreated cells. However, fewer CPE, including giant multinucleated cells, could be seen in cells transfected with 50 nM ICP4-targeting siRNA, and even fewer in the cells transfected with 100 nM ICP4-targeting siRNA.

ICP4-siRNA decreases ICP4 expression in HSV-1-infected RPEs

First, the ICP4 protein expression was observed by indirect immunofluorescence in HSV-1 infected cells. Compared with the cells transfected with mock siRNA or without siRNA, the intensity of immunostaining for ICP4 dramatically decreased in the RPEs transfected with ICP4-siRNA at 24 h p.i. (Fig. 4a). Under a higher magnification microscope, we found that the ICP4 protein was mainly localized in RPE nuclei, where it took part in the transcription activity. There was clearly less staining of ICP4 in 100 nM siRNA transfected cells than in 50 nM siRNA transfected cells.

The effect of siRNA on the targeted ICP4 protein was also detected by Western blot using antibodies against ICP4 (Fig. 4b). At 24 h p.i., the expression of ICP4 decreased more in the lysate of RPEs transfected with ICP4-siRNA than in siRNA untreated cells ($P < 0.05$). Similarly, there was no inhibition of ICP4 expression in HSV-1-infected cells that were transfected with the mock siRNA. Although the ICP4 protein tends to have lower expression in 100 nM siRNA-treated cells compared with that in 50 nM siRNA, the statistical analysis showed no significant differences ($P > 0.05$, Fig. 3c).

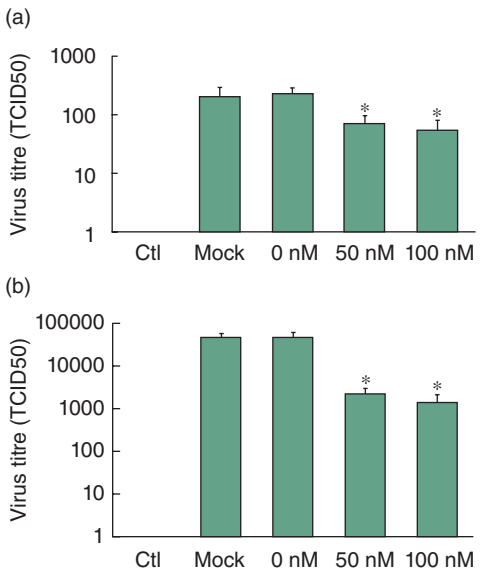

Figure 2. Quantitative viral titre in retinal epithelial cells (RPEs) transfected with small interfering RNA (siRNA). RPEs were infected with MOI 0.1 of herpes simplex virus type 1 (HSV-1) for 2 h, rinsed with phosphate-buffered saline and then transfected with siRNA. At 12 h (a) or 24 h (b) p.i., samples were harvested for virus titre assay. Every data point is the average from three independent experiments. The viral replication was significantly decreased in RPEs transfected with siRNA (50 nM and 100 nM). *$P < 0.05$. These average data were obtained from at least three independent experiments.

Decreasing expression of VP16 mRNA in ICP4-siRNA-treated RPEs

VP16, a transcription activator in HSV-1 lytic infection, is involved in the activation of immediate early genes, including ICP4.[2,34] In order to validate the interfered effects of siRNA on viral replication, VP16 was determined by RT-PCR (Fig. 5). After RPEs were infected with HSV-1 (MOI 0.1) 2 h p.i., the siRNAs were transfected. After culturing up to 24 h p.i., the expression of VP16 decreased in the cells transfected with siRNA compared with siRNA untreated cells ($P < 0.05$). The mock siRNA showed no effects on VP16 expression.

ICP4-siRNA decreases ICP4 expression in HSV-1-infected HCE, HTM and Vero cells

After HCE, HTM and Vero cells were infected by HSV-1 of MOI 0.1, followed by transfected with 100 nM siRNA, the ICP4 protein expression was analyzed by Western blot (Fig. 6). Compared with the cells transfected with mock or without siRNA, the expression of ICP4 dramatically decreased in the HCE (Fig. 6a,b), HTM (Fig. 6c,d) and Vero cells (Fig. 6e,f) transfected with ICP4-siRNA at 24 h p.i.

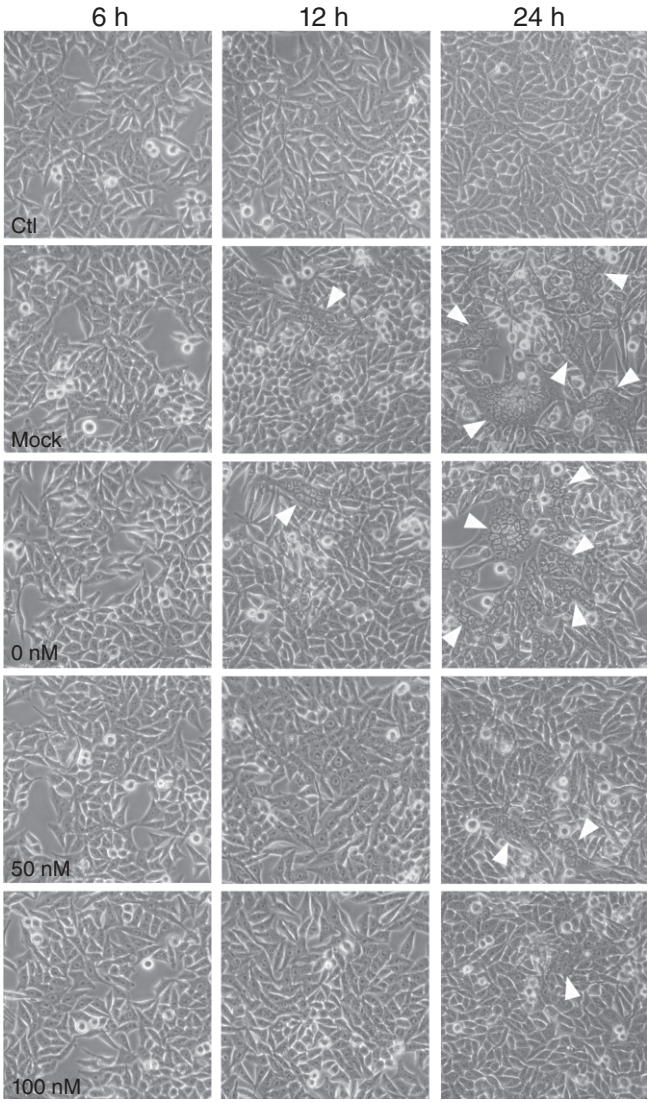

Figure 3. Imaging of herpes simplex virus type 1 (HSV-1) replication in cultured retinal epithelial cells (RPEs). RPEs were infected with MOI 0.1 of HSV-1. After 2 h, infected cells were transfected with small interfering RNAs (siRNAs). The cellular changes were imaged at 6 (left), 12 (middle) and 24 h (right) post-infection. Cytopathic effect (CPE) clearly increased, and many giant multinucleated cells could be seen in the cells treated with mock siRNA and cells untreated with siRNA (arrow); fewer CPE and giant multinucleated cells could be seen in cells transfected with ICP4-targeting siRNA.

Discussion

HSV-1, as a major pathogen to human beings, can cause a variety of diseases and can even be life-threatening. As ICP4 is an early key transcription activator in HSV-1 replication and target of HSV-1 miR-H6, we began this study with the goal of inhibiting HSV-1 replication by siRNA targeting to viral ICP4. We showed that ICP4-targeting siRNA significantly suppressed the expression of ICP4 and

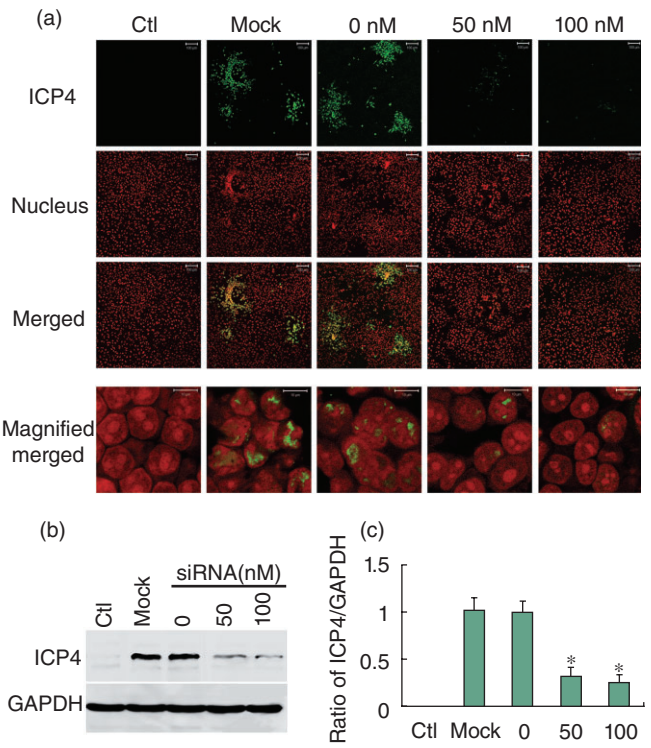

Figure 4. Small interfering RNA (siRNA) targeted against infected-cell polypeptide 4 (ICP4) inhibited ICP4 protein expression in retinal epithelial cells (RPEs). RPEs were infected with MOI 0.1 of herpes simplex virus type 1 (HSV-1). At 2 h p.i., cells were transfected with transfection reagent (Lipofectamine 2000), either alone or mixed with mock siRNA or 50 nM or 100 nM ICP4-targeting siRNA. After 24 h of incubation, experiments were terminated. (a): The staining of ICP4 protein in RPEs. FITC labelled the antibody stained ICP4 (green, top) and propidium iodide dyed the nucleus (red, middle). Images of ICP4 and nucleus were merged (2nd to bottom, scale bar: 100 μm). At the bottom of (a), magnified merged images (scale bar: 10 μm). (b): The ICP4 band was determined by Western blot. (c): Quantitative analysis of bands in (c). Significant decreases of ICP4 in RPEs transfected with siRNAs (*$P < 0.05$, siRNA *vs.* mock siRNA or 0 nM siRNA). Each data point is the mean value of three independent assays. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

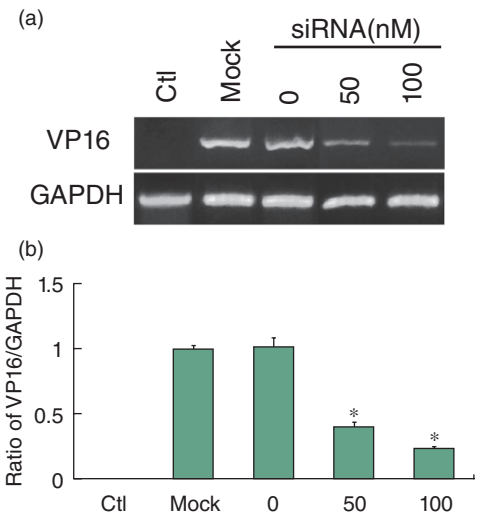

Figure 5. Reverse transcription polymerase chain reaction (RT-PCR) was used to observe the expression of VP16 in retinal epithelial cells (RPEs). RPEs were infected with herpes simplex virus type 1 (HSV-1) of MOI 0.1, and 2 h p.i. transfected with small interfering RNA (siRNA) and mock siRNA. At 24 h p.i., the expression of VP16 decreased in the RPEs transfected with siRNA, compared with those transfected with mock siRNA. GAPDH was used as an internal control. (a): Products of RT-PCR run on 1.5% agarose gel electrophoresis. (b): significant decreases of ICP4 in RPEs transfected with siRNA (*$P < 0.05$). Each data point is the mean value of three independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

decreased the viral levels in cultured RPEs. Less CPE and lower viral titre were seen in HSV-1-infected RPEs that were transfected with ICP4-siRNA compared with cells treated with mock siRNA or siRNA-untreated control cells. The tegument protein VP16 gene, a component of the virion that facilitates the immediate-early gene expression in a cascade of gene transcription,[11] was decreased in RPEs treated with ICP4-targeting siRNA, which mainly because of siRNA decreased HSV-1 replication. Furthermore, ICP4-siRNA downregulating the ICP4 expression was demonstrated in HCE, HTM and Vero cells (Fig. 6). These results suggested that using siRNA against ICP4 potently inhibited HSV-1 replication and may have the potential for clinical application.

ICP4 has crucial roles in HSV-1 lytic infection. It is the major transcriptional activator required for the activated expression of other HSV-1 genes.[2,10] Its functions of mediating the circularization of the HSV-1 genome, constitution of viral replication compartments, and formation of ICP8 microfoci in production infection have been demonstrated in the literature.[5,7] ICP4 also involves the delocalization of nucleolin, an abundant host nucleoli protein, from the nucleus to the cytoplasm, where it forms aggresomes for degradation.[35] In addition, ICP4 expression is regulated by the repressor element silencing transcription factor/neuronal restrictive silencer factor (REST/NRSF), which may play an important role in the establishment and/or maintenance of HSV-1 gene silencing during HSV-1 latent infection.[36]

RNAi technology has been used to silence gene expression by directly targeting a specific sequence of mRNA and has been reported to be an ideal tool for treatment of viral infectious diseases by silencing viral genes.[37,38] Many publications have described the inhibition of viruses, including human immunodeficiency virus type 1 (HIV-1), hepers simplex virus (HSV), hepatitis B virus (HBV) and hepatitis C virus (HCV), by targeting and silencing diverse viral genes as well as cell genes that are essential for virus

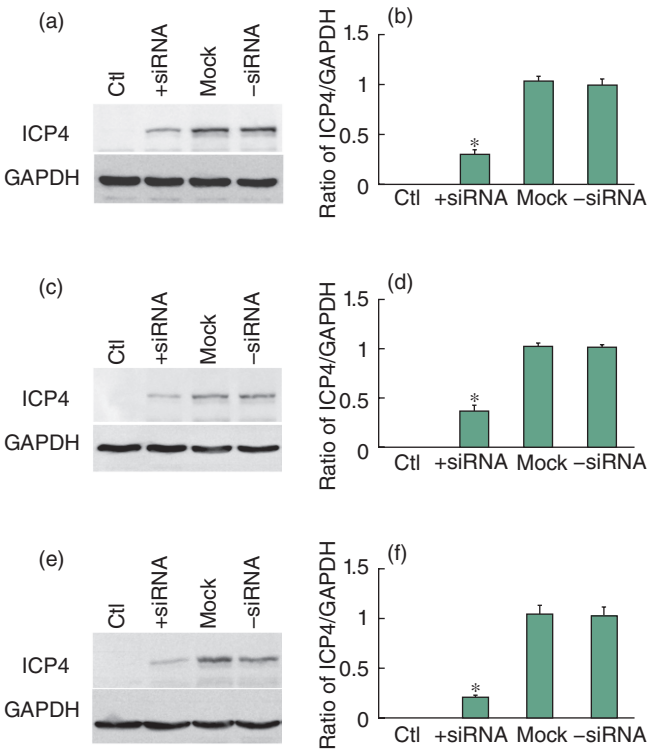

Figure 6. Infected-cell polypeptide 4 targeting small interfering RNA (ICP4-siRNA) decreases ICP4 expression in herpes simplex virus type 1 (HSV-1)-infected human corneal epithelial (HCE), human trabecular meshwork (HTM) and Vero cells. Cells were infected with HSV-1 of MOI 0.1 for 2 h and followed by transfected with or without ICP4-siRNA. At 24 h p.i., the expression of ICP4 protein decreased in the cells transfected with ICP4-siRNA, compared with those transfected with mock or without siRNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (a, c, e): The ICP4 protein band was determined by Western blot in HCE, HTM and Vero cells, respectively. (b, d, f): show quantitative analysis of bands with respective to those in (a), (c) and (e) ($*P < 0.05$). Cells were treated with siRNA (+siRNA or mock siRNA) or without siRNA (–siRNA). The control group (Ctl) consisted of cells without viral infection and siRNA transfection treatment. Each data is the mean of three independent assays.

replication.[27,39–41] Studies have applied RNAi to interfere with HSV-1 infection. Zhe *et al.*, for example, reported that synthetic siRNA silenced specifically the HSV-1 UL39 gene that codes ICP6 and inhibited HSV-1 replication in Vero cells.[25] There is also evidence that siRNA specific for glycoprotein E can suppress HSV-1 glycoprotein E expression and reproduce a gE-deletion mutant virus in keratinocytes.[27] In addition, using RNAi against cellular DNA ligases I, III and IV, Muylaert and colleague found that DNA ligase IV and its cofactor XRCC4 reduced HSV-1 production in human fibroblasts. On the other hand, RNAi-mediated depletion of neither DNA ligase I nor DNA ligase III appears to affect virus replication.[42] As RPEs are the target cells of HSV-1 in retina, we used RNAi to inhibit HSV-1 lytic infection in cultured RPEs. Our results show that using ICP4-siRNA can dramatically reduce HSV-1 replication in RPEs infected with viruses either before or after the transfection of siRNA.

In most previous studies, cells were transfected with siRNA and then infected with virus.[25,27,43] Under *in vivo* circumstances, however, HSV-1 infects the host before medical treatment is applied, so we need to find a way to treat the condition after viral infection, not before. We have reported that HSV-1 genes can be found in HSV-1-infected RPEs at 2 h p.i.[14] In the current study, we showed that the transfection of chemically synthesized ICP4-targeting siRNA could inhibit ICP4 protein expression in both pre- and post-HSV-1-infected RPEs (Fig. 1). In addition, inhibitory effects were related to the concentration of siRNA, such that 100 nM siRNA was somewhat more effective than 50 nM (Fig. 4). The inhibitory effects of siRNA concentration dependence have been reported in other virus infections, such as SARS coronavirus,[44] HCV,[41] and HIV.[39] In our study, however, neither 100 nM siRNA nor 50 nM siRNA could block all viral production, suggesting that the siRNA-delivery approach needs to be improved in the culture situation *in vitro*. Perhaps prolonging the periods of siRNA delivery or increasing the amount of valid siRNA in cells will suppress virus production more effectively.

Moreover, we designed three siRNAs and found that two siRNAs were successful as anti-HSV-1 agents, although one was more efficient (data not shown). Several studies have shown that clearance of the virus from a cell culture occurs only when the virus sequence is 100% identical to the siRNA,[38,41] and that multiple siRNAs that target one or more specific regions of the RNA sequences may limit the emergence of escape mutants and increase the virus-inhibition capacity.[41,45] Therefore, using multiple siRNAs to target ICP4 or other HSV-1 genes may inhibit virus production effectively. In addition, Liu *et al.* reported in Chinese that lentiviral plasmid pLKO-puro(r)-hU6-siRNA targeting ICP4 attenuates replication of HSV-1 in Vero cells,[46] which, in consist with our results, suggests that suppression of ICP4 can effectively inhibit production of HSV-1. However, considering the application RNAi *in vivo*, chemically synthesized siRNA can be more efficiently delivered into cells with less side effects,[47,48] and using direct introduction of synthetic siRNAs is most of the impending therapeutic applications based on RNAi propose.[47]

Recently, delivering genes or siRNAs by intravitreal or subretinal injection has been reported in treatment of retinal diseases. For example, siRNA anti-vascular endothelial growth factor (VEGF) has

been used in patients with neovascularization resulting from age-related macular degeneration, and stabilization or improvement in visual acuity has been observed.[49] Furthermore, studies reported that the subretinal injection of adeno-associated viral vectors encoding *RPE65* was safe and, in some cases, effective in patients with Leber's congenital amaurosis.[50] In addition, the use of intravitreal administration of siRNA was underwent clinical trials from different companies.[47] As a major pathogen in ophthalmic infections, HSV-1 can cause a variety of ocular diseases, ranging from mild to severe, and can even result in blindness.[21] We reveal that, *in vitro*, ICP4-siRNA could suppress HSV-1 reproduction in RPEs, which might be an effective way to inhibit HSV-1 replication in retinas using intravitreal injection.

In summary, we demonstrate that the synthesized ICP4-targeting siRNA exhibits excellent antiviral activity in inhibiting HSV-1 replication in RPEs. This finding provides a foundation for further studies on the use of ICP4-targeting siRNAs as effective antiviral agents against HSV-1 infection *in vivo*. Our study elucidates unique insights that may provide therapies to control retinal infection.

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