Targeting NECTIN-1 Based on CRISPR/Cas9 System Attenuated the Herpes Simplex Virus Infection in Human Corneal Epithelial Cells In Vitro

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Introduction

Herpes simplex keratitis (HSK) caused by herpes simplex virus (HSV), mainly by HSV-1, is an unignored cause of visual morbidity worldwide. As conservatively estimated, the global incidence of HSK is 1.5 million, among which 4000 new cases will have severe vision impairment or blindness each year.¹ In China, a multicenter research showed the prevalence of HSK was 0.11%.² HSK is characteristic with dendritic or geographic lesions in corneal epithelium in most initial cases, but it can also involve stromal and endothelial layers. As a neurotropic virus, HSV can easily maintain latency in the trigeminal ganglion and cornea for years; once induced by some stimuli, such as fatigue,
fever, or alcohol consumption, it may reappear again and again, and the recurrence of disease is often the reason resulting in severe vision impairment, even blindness. The antiviral agents such as acyclovir and ganciclovir are the most commonly prescribed for HSK, but these nucleotide analogues designed to block viral polymerase replication only work when the virus genome is actively replicating. Therefore, the herpes viruses that are good at being latent are hardly eliminated. These drugs are also unable to control the reactivation of viruses. Besides, in recent years, scientists found that the rate of drug resistance to acyclovir was rising. Therefore, looking for new therapies for HSK has been in demand.

Gene therapy has bloomed in recent years. In fact, the eye is an ideal target organ for gene therapy, owing to the ease to access or observe and its relative immune privilege. Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease (Cas) 9 systems have dominated in the field of gene therapy since their elaboration in 2012. From part of a bacterial adaptive immune system to a genome-editing tool, CRISPR/Cas9 systems only need a design of a 20–base pair guide sequence with the presence of required protospacer-adjacent motif to target specific genome loci and cause a double-sequence break, then initiating nonhomologous end-joining or homology-directed repair. For HSK, gene therapy that can directly edit virus genome is assumed to be the promising solution for eradicating latent herpesvirus. In recent years, the CRISPR/Cas9 system has been used for targeting some essential HSV genes, such as ICP0, UL7, UL8, UL19, UL27, UL29, and UL30. These studies all showed CRISPR/Cas9 application significantly reduced viral replication in vitro and in vivo. Among them, HELP (designated HSV-1–erasing lentiviral particles) has attained a breakthrough, which not only blocked HSV-1 replication but also modulated the viral reservoir in vivo. However, the problems of virus mutation, latent virus, repetitive application, and the low efficiency of gene editing arose in the gene therapy targeting the HSV genome, and in some cases, the CRISPR/Cas9 system only mediated weak gene editing. Thus, novel targets for the CRISPR/Cas9 system are still warranted.

The interactions between glycoprotein D (gD) on the HSV envelope and gD receptors on host cells, which allow for tight anchoring and trigger membrane fusion, are the crucial parts for viral entry. Several gD receptors on human cells have been identified, including herpesvirus entry mediator (HVEM), nectin cell adhesion molecule 1 (NECTIN-1), NECTIN-2, and 3-O-sulfated heparan sulfate proteoglycan (3-OS HS). Among them, NECTIN-1 and HVEM are expressed the most widely in human tissues and also are the most researched. In dermal infection of HSV, NECTIN-1 was proved to act as the more important and efficient gD receptor, while HVEM was an alternative choice. For HSV-1 infection of cornea, research in vitro or in vivo all demonstrated the importance of HVEM or NECTIN-1, especially the latter, for the invasion of virus. Besides, the internalization of NECTIN-1 from the cellular surface during HSV infection was believed to be part of the strategy of the virus to efficiently spread to uninfected cells. So, we suspected that if the interaction between gD and the crucial receptor on cornea, namely NECTIN-1, was blocked, the HSK might be lightened.

Hence, we employed the CRISPR/Cas9 system to edit the NECTIN-1 gene on human corneal epithelial cells (HCECs) to alleviate the HSV infection in vitro.

Materials and Methods

Cell Culture

Immortalized HCEC line was kindly provided by Prof. Shi Wei-yun (Shandong Eye Institute, Jinan, China) and Prof. Kaoru Araki-Sasaki (Osaka University, Osaka, Japan) and cultured as previously described. Human embryonic kidney 293 T cells (HEK293 T) were purchased from Cell Bank (CAS, Shanghai, China) and cultured in Dulbecco’s modified Eagle medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin.

Immunofluorescence Staining

HCECs were cultured on glass coverslips until 70% to 90% confluence and then fixed in 4% paraformaldehyde for 20 minutes. Next, cells were permeabilized with 0.03% Triton X-100, blocked with 3% bovine serum albumin, and stained with primary antibodies (anti–NECTIN-1, 1:100; Abcam, Cambridge, UK). Then, the samples were stained with secondary antibody (Alexa Fluor 488–labeled donkey anti-rabbit IgG, 1:1000; Thermo Fisher Scientific, Waltham, USA). Last, coverslips were observed through fluorescence microscopy (Leica Microsystems, Wetzlar, Germany).

Establishment of NECTIN-1 Knockdown Cells

Candidate single guide RNAs (sgRNAs) targeting human NECTIN-1 were designed on a Cas-designer tool, and seven sgRNAs were tested for the knockdown
efficiency in HEK293T cells. In short, these sgRNAs were separately cloned into lentiSaCRISPR v2 (#52961; Addgene, Watertown, USA) to get the lenti-Cas9-sgRNAs vectors. HEK293T cells were cotransfected with the lenti-Cas9-sgRNAs and the lentivirus packaging plasmids (MDLg, VSVG, and Rev). After 72 hours, different lentiviruses were collected. The HEK293T cells were infected with these lentiviruses, and the genomic DNA was extracted for Surveyor assays. Among the seven sgRNAs, two sgRNAs (sgRNA1: 5′-TGTGCCGATGAAGCCATACA-3′; sgRNA2: 5′-TTCCCAGCGTGAAGATCACCC-3′) were selected owing to their highest targeting efficiency.

HCECs were transduced with lenti-Cas9-sgRNA1, lenti-Cas9-sgRNA2, or lenti-Cas9, respectively, followed by puromycin selection for 7 days, to establish stably NECTIN-1 knockdown HCECs (NECTIN-1-KD1 and NECTIN-1-KD2) and the negative control (NC).

CCK-8 Assay

The three groups of HCECs were cultured in a 96-well plate (1 × 10^4 cells per well). After 24 hours, 10 μL CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) mixed with 100 μL culture medium was added to each well. After a 2-hour incubation, the absorbance was measured at 450 nm using a microplate reader (BioTek, Vermont, USA).

Western Blotting

Cellular proteins were extracted using cold RIPA lysis buffer (Beyotime, Shanghai, China) containing a protease inhibitor cocktail. Proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gels and then transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dried milk and then incubated with primary antibodies (anti–NECTIN-1, 1:1000; Abcam) overnight at 4°C, followed by incubation with horseradish peroxidase–conjugated goat anti-rabbit IgG secondary antibody (1:1000; Cell Signaling Technology, Danvers, USA). Chemiluminescence solution (MilliporeSigma, Burlington, USA) was used to reveal the bands. Representative blots from at least three independent experiments were shown.

Infection of HSV-1 on HCECs

HSV-1 with green fluorescent protein (GFP) (H129-G4), which encodes four copies of the enhanced GFP gene, was a gift from Prof. Luo Min-Hua.24 The three kinds of HCECs were infected with HSV-GFP at the multiplicity of infection (MOI) of 0.2 for 24 and 48 hours. The pictures of light microscopy and fluorescence microscopy were obtained simultaneously through the Live Cell Imaging System (Leica Microsystems) at different time points.

Flow Cytometry

HCECs were seeded in 6-well plates (2 × 10^5 cells/well). After 24 hours of HSV-GFP infection, cells were collected and resuspended in phosphate-buffered saline, and flow cytometry was performed on a Beckman Coulter MoFlo XDP (Brea, USA) for detecting GFP intensity. The percentages of GFP-positive cells were compared among the three kinds of cells.

DNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Dusseldorf, Germany). Quantitative real-time polymerase chain reaction (PCR) was subsequently conducted for quantifying HSV DNA on an ABI ViiA 7 Real-Time PCR System (Thermo Lifetech, Waltham, USA) using the QuantiNova SYBR Green PCR Kit (QIAGEN). Primer sequences are as follows: HSV-F, CTCATCAAGGGCGTGGAT, and HSV-R, TCGTAAAACAGCGTGAT, and HSV-R, TCGTAAAACAGCGTGAT.

Statistical Analysis

All quantitative data were expressed as means ± standard deviations of at least three independent experiments. Multiple groups comparisons were conducted through one-way analysis of variance followed by the Bonferroni test. P < 0.05 was considered significant. All the calculations were performed with SPSS 22.0 (IBM Corp., Armonk, NY, USA).

Results

CRISPR/Cas9 System Successfully Knocks Down the NECTIN-1 of HCECs

The result of immunofluorescence staining certified the high expression of the NECTIN-1 in the cytoplasm of normal HCECs (Fig. 1A). We designed seven candidate sgRNAs targeting the human NECTIN-1 gene. Among them, two sgRNAs, namely sgRNA1 and sgRNA2, were selected for their highest efficiency, and they both targeted the genome sequence encoding exon 2 of human NECTIN-1 (Figs. 1B, C). After the
Figure 1. Editing the NECTIN-1 using CRISPR-Cas9 in human corneal epithelial cells. (A) Immunofluorescence staining of the NECTIN-1 protein (green) in HCECs; nuclear DNA was counterstained with DAPI (blue). (B) The target sequences in the NECTIN-1 locus. The sgRNA target sequences and the protospacer-adjacent motif (PAM) sequence are shown in blue and red, respectively. (C) Surveyor assays performed at the targeting NECTIN-1 locus with genomic DNA from HRK-T93 cells transfected with lenti-Cas9-sgRNAs. (D) The NECTIN-1 proteins in the HCECs transduced with the lenti-Cas9-gRNA1 (KD1), lenti-Cas9-gRNA2 (KD2), and the lenti-Cas9 (NC) determined by Western blotting.

transfection of HCECs with lenti-Cas9-sgRNA1, lenti-Cas9-sgRNA2, or lenti-Cas9 separately and the following puromycin selection, the two stable NECTIN-1 knocking-down HCEC cell lines (NECTIN-1-KD1 and NECTIN-1-KD2) were successfully established. The efficiency was confirmed by Western blotting, using HCECs transfected with lenti-Cas9 as the control (NC) (Fig. 1C). The cell morphology and growth rate of the three groups remained the same (Supplementary Fig. S1).

Knocking Down the NECTIN-1 Increases the Cell Survival and Decreases HSV Infection

Thanks to the HSV-GFP (H129-G4) kindly provided by Prof. Luo Min-Hua,24 the HSV infection can be easily and intuitively observed and measured. Twenty-four and 48 hours after HSV-GFP infection at the MOI of 0.2, compared with NC, both the NECTIN-1-KD1 and the NECTIN-1-KD2 groups showed substantially better cell survival, cell morphology, and less virus infection, especially the NECTIN-1-KD1 group (Fig. 2).

After 24-hour HSV-GFP infection, flow cytometry results detected the significant decrease of GFP-positive cells in the NECTIN-1-KD1 and NECTIN-1-KD2 groups compared with the NC group. The NECTIN-1-KD1 group had almost an 85% reduction in HSV infection (Fig. 3A). At the same time, the relative HSV-DNA expression measured by quantitative PCR exhibited similar trends, with the NECTIN-1-KD groups only reaching 30% of the amount in the NC group (NC versus NECTIN-1-KD1: \( P = 0.000 \); NC versus NECTIN-1-KD2: \( P = 0.000 \); NECTIN-1-KD1 versus NECTIN-1-KD2: \( P = 1.000 \)) (Fig. 3B).

In summary, all the results above demonstrated that the knockdown of NECTIN-1 via the CRISPR/Cas9
Targeting NECTIN-1 through CRISPR/Cas9 In Vitro

Figure 2. NECTIN-1 knockdown by the CRISPR/Cas9 system increased the cell survival of HCECs. (A) Twenty-four hours after the HSV-GFP (green) infections at the MOI of 0.2, with light microscope and fluorescent microscope photographs of the NC-HCECs, NECTIN-1-KD1, and the NECTIN-1-KD2 HCECs. (B) Forty-eight hours after the HSV-GFP (green) infections at the MOI of 0.2, with light microscope and fluorescent microscope photographs of the NC-HCECs, NECTIN-1-KD1, and the NECTIN-1-KD2 HCECs.

system had a remarkable protective effect against HSV infection.

Discussion

Corneal blindness is the third leading cause of blindness worldwide, of which HSK is a leading cause in developing countries.25 Clearing latent virus and preventing recurrence are the biggest difficulties in eliminating HSV infection, and gene therapy is believed to be the only promising cure. So far, the research on the CRISPR/Cas9 system to treat ocular disease has made great advances, particularly for retinal diseases, but what should not be forgotten is that it is easier to access, operate on, and observe the cornea than the retina. In recent years, some work has been done to target the HSV genome by the CRISPR/Cas9 system in vitro, and it worked while the problems of outgrowth of resistant viruses and inability to target quiescent HSV arose.9,10,26 Compared to targeting viral genome, targeting virus receptors is a more direct and valid strategy without the worry of resistant virus, latent virus, and repetitive application on every recurrence. Blocking CCR5, which is the predominant coreceptor for human immunodeficiency virus (HIV) cell entry to treat HIV infection, is a successful example. Gene therapy through CRISPR/Cas9 has been demonstrated to be an effective way to eradicate HIV in vitro and in vivo.27–30 Therefore, even though our team has made a big stride in CRISPR/Cas9 targeting the HSV genome,12 we would like to discover more possibilities to eliminate ocular HSV.

As far as we know, this was the first study applying the CRISPR/Cas9 system targeting HSV receptors on the host cells against HSV infection. All the results in this study illustrated that the effective knockdown of NECTIN-1 on HCECs via the CRISPR/Cas9 system worked while the problems of outgrowth of resistant viruses and inability to target quiescent HSV arose.9,10,26 Compared to targeting viral genome, targeting virus receptors is a more direct and valid strategy without the worry of resistant virus, latent virus, and repetitive application on every recurrence. Blocking CCR5, which is the predominant coreceptor for human immunodeficiency virus (HIV) cell entry to treat HIV infection, is a successful example. Gene therapy through CRISPR/Cas9 has been demonstrated to be an effective way to eradicate HIV in vitro and in vivo.27–30 Therefore,
Figure 3. NECTIN-1 knockdown by the CRISPR/Cas9 system decreased the HSV infection of HCECs. (A) Twenty-four hours after the HSV-GFP infections at the MOI of 0.2, the typical flow cytometry results demonstrate the GFP fluorescence intensity of the NECTIN-1-KD1, NECTIN-1-KD2, and NC-HCECs. Blank control: NC-HCECs without the HSV-GFP infection. (B) Twenty-four hours after the HSV-GFP infections at the MOI of 0.2, the relative HSV-DNA expression was measured by quantitative PCR of the three kinds of HCECs (NC versus NECTIN-1-KD1: \( P = 0.000 \); NC versus NECTIN-1-KD2: \( P = 0.000 \); NECTIN-1-KD1 versus NECTIN-1-KD2: \( P = 1.000 \)). *** \( P < 0.001 \).

Demonstrated the attenuation of corneal infection. However, no research has tried to knock down NECTIN-1 on the host cell by the CRISPR/Cas9 system, and hence, we chose human NECTIN-1 as the target.

NECTIN-1 is a calcium-independent cell-cell adhesion protein that plays a role not only in the organization of intercellular junctions but also in polarization, differentiation, proliferation, and cell movement in several cell types.\(^3\)\(^{13}\) Except for the cornea, it is also the main receptor to HSV entry in the retina, derma, and brain. However, most HSV entry to the human eye is through the receptors on the ocular surface, as epithelial keratitis is the most common subtype of HSK.\(^3\)\(^{25}\) Although NECTIN-1 is expressed in the corneal epithelium, endothelium, and retinal pigment epithelium,\(^3\)\(^{4}\)\(^{5}\) we believed gene editing on the corneal epithelium was the most efficient method against ocular HSV infection.

The limitation of this study is that we did not detect the protective effect of knockdown of NECTIN-1 on HSV recurrence. HSV travels via the retrograde axonal ganglion to the TG and stays latent following the primary infection, waiting for reactivation. So far, recurrent HSV infection models have been established only in neural cells in vitro or in vivo, and the pattern of recurrent HSK makes it more suitable to be researched in vivo. Maybe in our future work, the effect of the CRISPR/Cas9 system targeting Nectin-1 on recurrent...
Human sterile keratitis (HSK) will be investigated. In addition, although the growth rate, growth pattern, and cell morphology did not change much after the knockdown of NECTIN-1 on HCECs, the safety issue for gene editing by the CRISPR/Cas9 system still warrants long-term investigation, especially in the future practice in vivo.

In conclusion, by using the CRISPR/Cas9 system, we successfully knocked down the NECTIN-1 expression in vitro, which alleviated the HSV infection in HCECs, offering a promising therapy for HSK.

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References


