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In vitro inhibition of classical swine fever virus replication by siRNAs targeting N^{pro} and *NS5B* genes

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Abstract

Classical swine fever (CSF) is a highly contagious disease of pigs, which causes important economic losses worldwide. In the present study, the specific effect of RNA interference on the replication of CSF virus (CSFV) was explored. Three species of small interfering RNA (siRNA), targeting different regions of CSFV N^{pro} and *NS5B* genes, were prepared by *in vitro* transcription. After transfection of PK-15 cells with each of the siRNAs followed by infection with CSFV, the viral proliferation within the cells was examined by indirect immunofluorescence microscopy. At 72 h post-infection, only a few siRNA-treated cells were positive for viral antigen staining, while most untreated virus-infected cells were positive. Treatment with the siRNAs caused a 4–12-fold reduction in viral genome copy number as assessed by real time RT-PCR. Transfection with the siRNAs also suppressed the production of infectious virus by up to 467-fold as assessed by $TCID_{50}$ assay. These results suggested that the three species of siRNAs can efficiently inhibit CSFV genome replication and infectious virus production, with the inhibition persisting for 72–84 h.

Keywords: CSFV; Replication; siRNA

1. Introduction

RNA interference (RNAi) has been recognized as a mechanism for repression of gene expression induced by small interfering RNAs (siRNAs) of about 21–23 nt (Fire et al., 1998; Elbashir et al., 2001). Since its discovery, RNAi has been regarded by virologists as a promising method for the suppression of viral infection (Cullen, 2002; Pomerantz, 2002; Kitabwalla and Ruprecht, 2002), and has been successfully applied to inhibit the replication of some human and other animal viruses, including HIV-1 (Pomerantz, 2002; Kitabwalla and Ruprecht, 2002; Lee et al., 2002; Jacque et al., 2002), hepatitis C virus (Kapadia et al., 2003; Wilson et al., 2003), poliovirus (Gitlin et al., 2002), foot-and-mouth disease virus (Chen et al., 2004), porcine reproductive and respiratory syndrome virus (He et al., 2007) and porcine transmissible gastroenteritis virus (Zhou et al., 2007), both *in vitro* and *in vivo*.

Classical swine fever (CSF) is a highly contagious disease of pig caused by infection with CSF virus (CSFV), and causes great economic losses in the pig industry worldwide. CSFV, along with bovine viral diarrhea virus (BVDV) and border disease virus (BDV), belongs to the genus *Pestivirus* of the family Flaviviridae (Heinz et al., 2004). Its genome consists of a single-stranded (+) sense RNA of about 12.5 kb with a single large open reading frame (ORF), encoding a polyprotein. After translation, the polyprotein is cleaved into viral structural and non-structural peptides which are from N- to C-terminus, N^{pro}-C-E^{rns}-E1-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Meyers et al., 1989; Tautz et al., 1997; Elbers et al., 1996; Ruggli et al., 1996). N^{pro} is a cysteine protease, a key enzyme for processing the polyprotein, which is encoded by the 5′ terminus of ORF (Rümenapf et al., 1998). NS5B is a type of RNA-dependent RNA polymerase responsible for viral RNA replication (Zhang et al., 2005; Xiao et al., 2006). In order to explore a new approach for prevention and therapy of classical swine fever, *in vitro*-transcribed siRNA molecules were studied for their inhibitory effects on CSFV replication. Our results show for the first time that 21 nt siRNAs are able to specifically and efficiently inhibit CSFV replication *in vitro*. This study provides not only an experimental basis for the development of a new anti-CSFV strategy, but also for a new approach to the study of CSFV infection and replication.

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2. Materials and methods

2.1. Cells, virus, and sera

The pig kidney cell line PK-15 (80 passages) and CSFV strain Shimen were obtained from the Institute of Veterinary Drug Control, China. Positive anti-CSFV serum and negative control serum were prepared as described previously (Yu et al., 2001).

2.2. CSFV siRNA preparation

Sequences from the N^{pro} and *NS5B* genes (GenBank accession AF092448) were scanned for the signature sequence AA-N_{19}. All 21 nt candidate sequences in these two genes were searched against the GenBank database using Blast N, to avoid similar sequences in the pig genome. Candidates were further analyzed with Vector NTI 3.0 software (Informax, Piscataway, NJ) for significant secondary structure, GC content and melting temperature. Two sequences, siN1 and siN2, were chosen as siRNA for N^{pro}, and one, si5B1, for *NS5B*. The reverse sequence of siN1 was used as a negative siRNA control (siCtrl). The T7 promoter sequence was used for *in vitro* transcription. For each sequence, oligo DNA was synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. The sequences are

- T7p: GGATCCTAATACGACTCACTATA
- 5B1S: AAGACGTCCCTCTTCTCATTCTATAGTGAGTCGTATTAGGATCC
- 5B1AS: AGAATGAGAAGAGGGACGTCTATAGTGAGTCGTATTAGGATCC
- N1S: AAGCCTGTCACCCTACCTATCCTATAGTGAGTCGTATTAGGATCC
- N1AS: AGGATAGGTAGGGTGACAGGCTATAGTGAGTCGTATTAGGATCC
- N2S: AAGCTAATCCACTTCAGGGTTCTATAGTGAGTCGTATTAGGATCC
- N2AS: AGAACCCTGAAGTGGATTAGCTATAGTGAGTCGTATTAGGATCC
- CtrlS: AAGCCTGTCACCCTACCTATCCTATACCTAGGATTATGCTGAGTG
- CtrlAS: CACTCAGCATAATCCTAGGTATAGGATAGGTAGGGTGACAGGC

where S is the sense strand, AS is anti-sense strand, and Ctrl is the control siRNA sequence.

The T7 promoter primer T7p was annealed to each of the synthesized siRNA sense or anti-sense oligo DNAs. The T7 primer was subsequently extended conventionally with DNA polymerase to obtain double-stranded template DNA. The template DNA was purified by ethanol precipitation, resuspended in 50 μL DNase- and RNase-free water and then transcribed *in vitro* to produce a sense or an anti-sense RNA by using T7 RiboMAX™ Express RNAi System (Promega), following the manufacturer's instructions. After destruction of the DNA template by incubation with 1 μL RNase-free DNase at 37 °C for 30 min, the sense RNA strand and its corresponding anti-sense RNA were mixed and incubated at 70 °C for 10 min. Gradual reduction to room temperature permitted annealing to form siRNA, which was precipitated with isopropanol, washed with ethanol, and air-dried. The resulting siRNAs, siN1, siN2, si5B1, and siCtrl, were resuspended in 100 μL water and examined by electrophoresis. Concentrations were determined with the GeneQuant RNA/DNA Calculator (Pharmacia Biotech, Piscataway, NJ).

2.3. Cell culture and transfection

PK-15 cells in MEM + 10% fetal bovine serum (FBS) without antibiotics were seeded in 24-well plates at 10^4 cells/well, and incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. When cells were 30–50% confluent, siRNA was introduced using X-tremeGene siRNA transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's protocol. Briefly, 2.5 μL transfection reagent and 0.5 μg siRNA was added to each well and incubated for 4 h. The cells were then washed with MEM and further cultured for 20 h in MEM + 2% FBS prior to viral infection. The siRNA-treated cells were infected with 500 $TCID_{50}$ of CSFV (strain Shimen) and cultured for the various time periods indicated. Infected cells were visualized by indirect immunofluorescence assay. The copy number of CSFV genomic RNA was determined by real time RT-PCR and infectious virus production was determined by the $TCID_{50}$ assay.

2.4. Indirect immunofluorescence assay (IFA)

Cell monolayers in wells were fixed with 80% cold acetone for 30 min, washed three times with PBS (pH 7.4), and incubated for 1 h with pig anti-CSFV serum (1:100) at 37 °C in a humidified box. Cells were then washed three times with PBS and incubated with FITC-conjugated rabbit anti-pig IgG (Sigma; 1:60) for 1 h. After washing, cells were examined and photographed with a Zeiss Axioskop 40 fluorescence microscope.

2.5. Determination of virus genome copy

2.5.1. Primers and probe

In order to use full-length NS5B gene as a quantitative RT-PCR standard, primers were designed for CSFV strain Shimen using Vector NTI 3.0 (Informax, USA). Selected primer sequences were GACAGATCTAGTAATTGGGTGATGCAAGAAG (NS5B FP primer), and GAAGTCGACTAC CCCTCTCCCTATCAGGGTCATC (NS5B RP primer). Three additional primers were synthesized for quantification of the CSFV genome in real-time PCR: GGGATGATGGTCTCCTGATCAC (rFP primer), CATCAAATTGGTAGGCCACTTTC (rRP primer), and 5′-FAM-CCTTGCTCGCGAATTTCTCACCGATAMRA-3′ (TaqMan probe Prob5B). The target region of real time RT-PCR was nt 1,1253–1,1390 of NS5B.

2.5.2. Construction of NS5B gene plasmid

Total RNA of CSFV-infected cells was extracted with Trizol LS Reagent (Invitrogen, Carlsbad, CA) and then applied

as a template for the synthesis of the first cDNA strand of the NS5B gene using the ThermoScript RT-PCR SYSTEM (Invitrogen) with AMV reverse transcriptase and NS5BRP primer, according to the manufacturer's instructions. Afterwards, PCR was used to amplify the NS5B gene with NS5BFP and NS5BRP primers under conditions of 94 °C for 2 min, then 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 120 s, with a final extension at 72 °C for 7 min. The PCR product was gel-purified using the Agarose Gel DNA Extraction Kit (Roche) and then cloned into pGEM-T vector (Promega, Madison, WI). The resulting plasmid, pT-NS5B, with the correct sequence confirmed by direct sequencing, was selected as a quantitative standard for the determination of the viral RNA copy number. The plasmid was prepared using the Wizard Plus SV Minipreps DNA Purification System (Promega), aliquoted and stored at −80 °C after the determination of its concentration by the GeneQuant RNA/DNA Calculator (Pharmacia Biotech).

2.5.3. Real-time PCR analysis

For quantitative analysis of the CSFV genome, 100 ng total RNA of CSFV-infected cells was mixed with 1 μL rRP primer, heated to 65 °C for 5 min and chilled on ice for 2 min. To this primer-template mix was then added 5× buffer (4 μL), 10 mmol/L dNTP (1 μL), RNasin (1 μL), AMV reverse transcriptase (1 μL, Promega) and water to a total volume of 20 μL. The reaction mixture was incubated at 42 °C for 45 min, followed by inactivation of the reverse transcriptase at 75 °C for 15 min. Real-time PCR was performed with the ABI PRISM® 7000 Sequence Detection System using a QuantiTect™ Prob PCR Kit (Qiagen, Germany) under the conditions of 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing, and extension at 60 °C for 30 s. The quantitative standard curve for the determination of CSFV genome copy number was created by real-time PCR of standard plasmid pT-NS5B preparations at serial dilutions of 600, 1200, 2400, 4800 and 9600 copies/μL. The specificity of the real-time PCR was confirmed by sequencing of the product.

2.6. Titration of virus

CSFV cultures in siRNA-transfected cells were serially diluted 10-fold from 10^{-1} to 10^{-6}, added to PK-15 cells at 30–50% confluency in 96-well plates as described above. Each dilution was added to four wells and the cultures were examined for viral growth by IFA at 72, 84, 96 and 120 h post-infection. Incubation was at 37 °C in a 5% CO_2 atmosphere. $TCID_{50}$ values were calculated by the Kärber method (Kärber, 1931).

3. Results

3.1. Examination of siRNA effect by IFA

To study the inhibitory effects of RNA interference on CSFV replication, the level of viral antigen produced in PK-15 cell was examined by IFA using anti-CSFV serum after siRNA transfection and viral infection. At 72 h post-CSFV infection, most PK-15 cells receiving siCtrl or mock transfection exhibited bright green fluorescence in the cytoplasm, indicating that most cells in these two controls were producing virus (Fig. 1B and C). By contrast, only a few cells in wells receiving siN1, siN2 or si5B1 transfection displayed green fluorescence, indicating that most of the cells were effectively protected by the siRNA and resisted viral infection (Fig. 1D–F).

3.2. Examination of siRNA effect by RT-PCR

In order to quantify the effect of siRNA on viral replication, the viral genome copy number was determined by real-time PCR, using serially diluted plasmid pT-NS5B as a standard. The R_2 value of the standard curve was 0.99 and the average amplification efficiency E was 0.991, calculated from the formula $E = 10^{-1/\text{slope}} - 1$. These data indicate that the real-time PCR was highly reliable in the assay, and the results from amplification of the siRNA samples showed that at 72 h post-viral

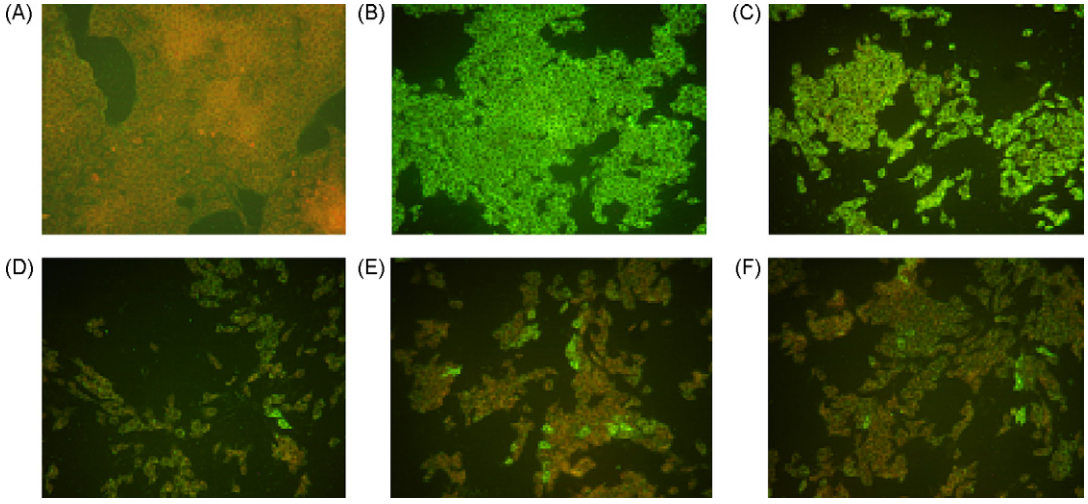

Fig. 1. Protective effect of siRNA against CSFV infection. The viral infection in PK-15 cells was examined by indirect immunofluorescence. (A) Mock transfection (stained with CSFV-negative serum); (B) mock transfection (stained with CSFV-positive serum); (C) siCtrl transfection; (D) siN1 transfection; (E) siN2 transfection; (F) si5B1 transfection.

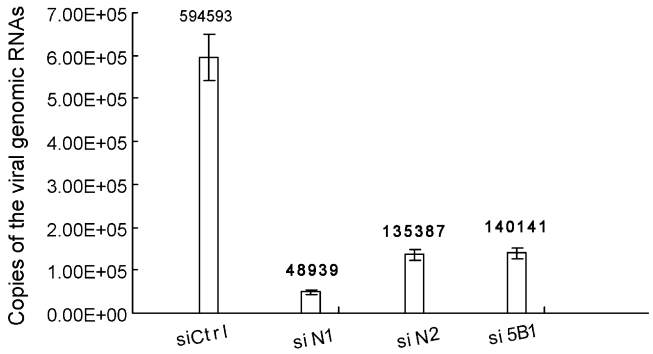

Fig. 2. Reduction of viral genome copy number by siRNA treatment in PK-15 cells at 72 h post-infection. The CSFV genome copy numbers are the means of three repeat experiments.

infection, the typical C_t value for amplification of CSFV RNA from cells treated with siCtrl was 21.46, compared with 25.05, 23.59 and 23.54 in siN1-, siN2- and si5B1-treated cells, respectively, indicating suppression of CSFV replication in the latter groups. Sequencing showed that the fragment amplified was the expected part of NS5B gene, thereby demonstrating the specificity and reliability of the analysis. As shown in Fig. 2, the copy number of the viral genome per nanogram of total RNA calculated from the standard curve was 1.19×10^5 copies/ng of the viral genome in the total RNA from siCtrl-treated cells, while there were 9.8×10^3, 2.7×10^4, and 2.8×10^4 copies/ng viral genome in total RNA from cells treated with siN1, siN2 and si5B1, respectively, corresponding to reductions of 91.8%, 76.3% and 76.5%, respectively.

3.3. Examination of siRNA effect by infectious virus assay

The $TCID_{50}$ assay was performed to examine the effect of siRNA on production of viable virus, and the results (see Fig. 3) showed that in control cells transfected with siCtrl CSFV titers reached $10^{4.33}$ $TCID_{50}$/mL at 60 h post-infection, similar to that obtained from mock transfection (data not shown), This was followed by a gradual decrease in titer from 72 to 120 h post-infection. In contrast, titers at 60 h post-infection were $10^{1.67}$, $10^{2.83}$ and $10^{2.58}$ mL^{-1} for cells transfected with siN1, siN2 and si5B1, respectively, corresponding to 467-, 30.6- and 55.2-fold reductions by comparison with siCtrl-transfected cells. The

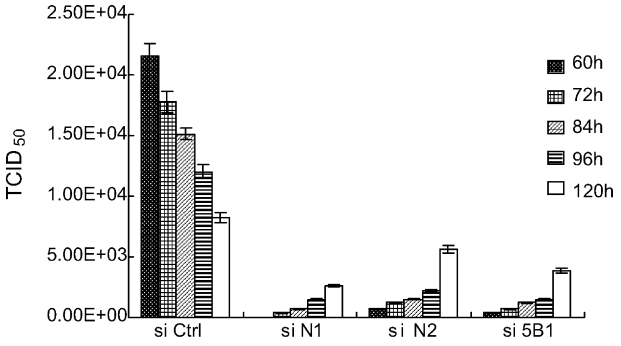

Fig. 3. Inhibition of virus production in siRNA-treated cells. $TCID_{50}$ values are the means of three repeat titrations at the time points indicated.

data indicate that the siRNAs markedly inhibited infectious virus production for the first 2.5 days, but thereafter the extent of inhibition decreased.

4. Discussion

In most studies, inhibition of viral proliferation has been accomplished by siRNA produced within the cells by transcription of expression constructs. The *in vivo* expression of siRNA for *LTR*, *vif* and *nef* genes of HIV-1 in Magi and PBLs cells mediated by specifically constructed plasmids showed that HIV-1 replication was decreased by 20-fold (Jacque et al., 2002). Jia and Sun (2003) achieved a greater than 43-fold inhibition of DNA virus MHV-68 replication by siRNA. In the present study we designed three siRNA molecules of 21 nt against genes N^{pro} and *NS5B*, two key genes related to CSFV replication, and the results showed that these three siRNAs were highly capable of inhibiting viral genomic RNA replication, viral antigen synthesis and infectious virus production with siN1 reducing the viral replication by up to 467-fold. The CSFV genome contains a single large ORF encoding a polyprotein (Meyers et al., 1989). Since N^{pro} is the ultimate N-terminal protease involved in the polyprotein processing the binding of siRNA to N^{pro} mRNA likely interfered with the translation and the subsequent processing of the viral polyprotein, thereby resulting in a significant inhibition of viral replication. Moreover, there are studies showing that it is essential that the sequence downstream of the internal ribosome entry site within the starting region of CSFV mRNA should be single-stranded for high efficiency translation (Rijnbrand et al., 2001; Fletcher et al., 2002). Consequently, since this part of the N^{pro} mRNA may not form a secondary structure, it is likely to be highly accessible to siRNA (Kretschmer-Kazemi Far and Sczakiel, 2003). *NS5B* encodes the RNA-dependent RNA polymerase, which binds to the 3′ non-coding region (NCR) of the viral RNA and initiates its replication. Binding to this part of the genome will cause the degradation of the entire viral RNA, likely reducing the viral replication. Based on this knowledge, satisfactory RNA interference results were obtained by siRNAs generated against both key genes, although siN1 exhibited a more potent effect than siN2 and si5B1. Our results are similar to those obtained by siRNA against the *NS5B* gene of hepatitis C virus (Kapadia et al., 2003; Wilson et al., 2003), which belongs to the same virus family as CSFV.

Knowledge of the kinetics of CSFV replication in cultured cells is critical for the determination of the sampling points for the estimation of siRNA effect in cells. Hence, the replication kinetics of CSFV strain Shimen in PK-15 cells was studied by IFA and $TCID_{50}$ assay prior to siRNA transfection, with results showing that the proliferation of virus peaked at 48–72 h post-infection (data not shown). Based on this consideration, viral growth in siRNA-transfected cells was determined at 60 h post-infection and determination of viral genome copy number and staining by IFA were conducted at 72 h post-infection.

Silencing of gene expression induced by *in vitro*-transcribed siRNA molecules in cultured cells is time-dependent, as shown in Fig. 3. Significant elevations in virus titers seen at 120 h post-

infection of siRNA-treated cells indicates that the effect of these siRNAs diminished after 96 h post-transfection. In order to further study the duration of the siRNA effect, siN1 was chosen since it exhibited the strongest inhibition as observed above. PK-15 cells were transfected with the same amount of siN1, then infected with CSFV as described above. IFA was used to determine virus replication at 72, 84, 96 and 120 h post-viral infection. The result showed that at 72 and 84 h post-infection, only a few cells were infected and at 96 h about half of the cells were infected, while at 120 h almost all cells were infected, as shown by positive staining (data not shown). These results indicate that effective inhibition by the siRNAs used lasted for 72–84 h.

RNAi is highly sequence-specific and requires that the target and targeting sequences have 100% identity. Pestiviruses are genetically variable. The overall interspecies sequence identity within the genus is less than 70%, and sequence identity within a species varies from 75% to 100%. This genetic variety makes it impossible to design a siRNA able to cross-inhibit all species. Lambeth et al. (2007) explored the RNAi of BVDV with siRNAs targeting the 5′NCR, *C*, *NS4B* and *NS5A*, and found that siRNAs targeting *C*, *NS4B* and *NS5A* could effectively inhibit viral replication. These latter regions are not conserved, however, which probably means that the siRNAs would not cross-inhibit all BVDV isolates. In order to evaluate the cross-inhibitory capabilities of the siRNAs studied, multiple alignments of N^{pro} and *NS5B* sequences of CSFV strains were conducted based on their availability in GenBank. Results showed that siN1, si5B1 and siN2 could cover 71% (20/28), 64% (18/28) and 60% (17/28) of CSFV strains, respectively (data not shown), indicating that more efforts should be done to search for siRNAs with the widest cross-inhibitory effects on a range of CSFV or BVDV strains.

Therapy for animal viral infections is an interesting possibility, although eradication is the worldwide policy, especially in developed countries, to prevent outbreaks of infectious diseases. Our study has demonstrated that RNAi has potential for the treatment of CSFV infection. This method may not completely inhibit viral growth, but it merits further animal studies to define its real therapeutic potential. The siRNAs in the present study had a quite long effect compared with those for RNAi of foot-and-mouth disease virus, which had an *in vitro* effect lasting for 42 h (Chen et al., 2004). The effect of siRNA can be enhanced by combined use of different siRNAs or by repeated application of siRNA at regular time intervals. If successful in clinical application, siRNA therapeutics could be used as a preventive approach for the protection of healthy pig populations close to the areas of CSF outbreaks. This concept might help to reduce viral transmission during the immunity gap between vaccination and the onset of protection, thereby gaining precious time for bringing outbreaks under control.

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