Veterinary Microbiology 142 (2010) 41–44

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

RNA interference targeting nucleocapsid protein (C) inhibits classical swine fever virus replication in SK-6 cells

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ARTICLE INFO

Keywords:
RNA interference
Small interfering RNA
Nucleocapsid protein (C)
Classical swine fever

ABSTRACT

The application of RNA interference (RNAi) strategy for controlling classical swine fever could become a promising alternative to the conventional eradication measures, as it was recently shown for foot-and-mouth disease (Chen et al., 2004), influenza (Ge et al., 2003), porcine reproductive and respiratory syndrome (He et al., 2007) and porcine transmissible gastroenteritis (Zhou et al., 2007). The use of synthetic siRNA which is corresponding to nucleotides 1130–1148 of the CSF virus strain Alfort, targeting the nucleocapsid protein (C) was investigated to show the inhibition of CSF virus replication. It could be shown that the virus titer of infected cells, which had been mock-transfected or transfected with control (non-silence) RNA were not affected. These data indicate that siRNA_253 is able to inhibit viral replication.

1. Introduction

RNA interference (RNAi) is a mechanism in eukaryotic cells, which inhibits gene expression. Responsible for the gene specific down regulation is a small interfering RNA (siRNA) of 21–23 nucleotides. siRNA is derived from long double-stranded RNA molecules cleaved into short fragments of ~20 nucleotides using enzyme Dicer. One strand of a short double-stranded RNA molecule is incorporated into the RNA-induced silencing complex (RISC). A complementary sequence to messenger RNA molecule base pairs with RISC resulting in the induction of cleavage by Agronaut, the catalytic component of the RISC complex. This process has been identified as the main defense mechanism against viral infection in plants (Gitlin and Andino, 2003; Lecellier and Voinnet, 2004). It remains unclear, though, whether RNAi is a natural antiviral mechanism in animals (Grubman and Santos, 2005). In mammals, RNAi has shown promising results in inhibiting the replication of several RNA and DNA viruses in animals (Haasnoot et al., 2003) such as foot-and-mouth disease virus (Chen et al., 2004), influenza virus (Ge et al., 2003), porcine reproductive and respiratory syndrome virus (He et al., 2007) and porcine transmissible gastroenteritis virus (Zhou et al., 2007). Recently, Xu et al. (2008) reported an inhibition of classical swine fever virus replication by siRNAs targeting *Npro* and *NS5B* genes.

Although classical swine fever (CSF) has been known for almost 200 years, it is still not eradicated and the improvement of eradication strategies is desirable. Various strategies for CSF eradication and control have been implemented in many countries, including stamping out, ban on import of live pigs (van Oirschot, 1999), and vaccination. Nevertheless, CSF is not eradicated all over the world.

CSF virus is a small enveloped RNA virus with a genome consisting of single-stranded RNA of positive polarity (Moormann et al., 1990; Thiel et al., 1991). Its single long open reading frame (ORF) is translated into 12 mature proteins (Thiel et al., 1991). The formation of the nucleocapsid protein (C)–RNA complex inside the virion suggests a protective function to the core protein (Thiel et al., 1991; Meyers and Thiel, 1996). According to Liu et al.

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doi:10.1016/j.vetmic.2009.09.041

(1998), the expressed nucleocapsid protein (C) can activate the human heat shock protein 70 promoter, and can suppress the SV40 early promoter. These findings indicate that the nucleocapsid protein (C) functions not only as a viral structural protein but also as a regulator of gene expression. Therefore, the inhibition of CSF virus C protein using RNAi could also down regulate virus gene expression. In this study, we show the influence on CSF virus replication using RNAi targeting to the nucleocapsid protein (C).

2. Materials and methods

2.1. Cells, virus and sera

Swine kidney cells (SK-6) were acquired from the veterinary research and development center, upper northeast. CSF virus Bangkok 1950 strain and anti-CSF virus serum were obtained from the National Institute of Animal Health of Thailand.

2.2. siRNAs (small interfering RNA)

Double-stranded RNA sequences, designated siRNA_253, were designed according to sequences of nucleocapsid protein (C) of CSF strain Alfort (Genbank accession number X87939) using Invitrogen Block-iT RNAi Designer and were synthesized by Invitrogen. The used sequence was 5′-GCAUGGGCGGUAAUAGCAAdTdT-3′. Double-stranded control (non-silence) siRNA of the following sequence UUCUCCGAACGUGUCACGUdTdT was purchased from Qiagen. Duplexes were resuspended in DEPC-treated water to obtain 20 μM solutions, heated at 90 °C for 1 min and incubated at 37 °C for 60 min prior to use.

2.3. Cell culture and cytotoxicity assays

SK-6 cells were cultured in modified Eagle's medium (MEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) (pH 7.4) without antibiotics (maintenance medium) at 37 °C under 5% CO_2 atmosphere. The siRNA transfection reagent (X-tremeGENE siRNA Transfection Reagent, Roche) and siRNA, were diluted in Opti-MEM at a ratio of 5:1 (transfection siRNA complex) according to the manufacture's recommendations. To determine the optimal concentration of transfection reagent and siRNA used in the experiment, cytotoxicity test of SK-6 in a 24-well plate was performed. At 50 percent confluency, 100 μl of transfection siRNA complex and 400 μl Opti-MEM were added to the cells. Maintenance medium was added instead of transfection siRNA complex after 4 h of incubation at 37 °C, 5% CO_2. Transfected SK-6 cells were then incubated for another 72 h under the same conditions. The cell morphology was observed microscopically to detect alterations, e.g. loss of monolayer, rounding, shrinking of the cells, granulation, and vacuolization in the cytoplasm. The cytotoxic concentration was determined as the maximal concentration of transfection siRNA complex that did not exert a toxic effect to SK-6 cells (Kudi and Myrint, 1999; Kujumgier et al., 1999).

2.4. siRNA transfection in CSF virus infected SK-6 cells

siRNA transfection was performed in a 24-well plate containing approximately 4×10^4 SK-6 cells according to the manufacturer's instructions. Briefly, 1-day-old cells (30–50% confluency) were infected with CSF virus at 5.65, 4.65 and 3.65 ($\log_{10}$ $TCID_{50}$/ml). Transfection siRNA complex at different concentrations was added to each well 24 h later and incubated for 8 h. The cells were then washed with MEM and further incubated in maintenance medium at 37 °C in a 5% CO_2 atmosphere for 4 days. Cell culture supernatant was collected after subjecting the plate to two cycles of freezing and thawing.

2.5. Virus titration

Serially 10-fold dilution of siRNA-transfected cells supernatants from 10^{-1} to 10^{-8} were added to 30–50% confluent SK-6 cells in 96 wells plates at 37 °C, 5% CO_2. Each dilution was added to 4 wells. Maintenance medium with 100 units/ml penicillin and 100 μg/ml streptomycin were used as culture medium. An indirect immunoperoxidase assay was performed to identify the remaining virus. $TCID_{50}$ values were calculated according the method of Kärber (1931).

3. Results

3.1. Cytotoxicity assays

Cytotoxicity test was performed to examine the effect of siRNA transfection reagent and siRNA on SK-6 cells. The results (Table 1) showed that at a siRNA transfection reagent (μl): siRNA (μg) ratio of 5:1, an amount of 10 and 5 μl transfection reagent caused cytotoxicity in SK-6 cells, whereas 3.75, 2.5, 1 and 0.5 μl caused no cytotoxicity in SK-6 cells.

3.2. Viable virus after CSF virus infected SK-6 were transfected

0.75, 0.5, 0.2 and 0.1 μg siRNA_253 and non-silence siRNA were used to transfect CSF virus infected SK-6 cells in order to investigate the effect on the production of viable virus. SK-6 cells were infected with three different infectious doses of CSF virus (5.65, 4.65 and 3.65 $\log_{10}$ $TCID_{50}$/ml). Transfection was carried out with siRNA_253, non-silence siRNA and mock 24 h after infection. Four days later cell supernatants were harvested and CSF virus was

Table 1
Cytotoxicity test of SK-6 at various concentrations of transfection reagent and siRNA.

Transfection reagent (μl)	siRNA complex (μg)		Cytotoxicity
	siRNA_253	Non-silencing siRNA	
10	2	2	Yes
5	1	1	Yes
3.75	0.75	0.75	No
2.5	0.5	0.5	No
1	0.2	0.2	No
0.5	0.1	0.1	No