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Inhibition of Bovine herpesvirus multiplication in MDBK cells by small interfering RNAs

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Summary. – Small interfering RNA (siRNA) mediated gene silencing is a promising approach in antiviral therapy. To investigate the antiviral effects of siRNAs on Bovine herpesvirus 1 (BoHV-1) multiplication, we designed and *in vitro* synthesized two siRNAs (siRNA-1 and siRNA-2) targeting the UL25 gene that is essential for BHV-1 multiplication. siRNA-1 and siRNA-2 inhibited the BoHV-1 multiplication in MDBK cells to a different extent, namely by 11% and 40%, respectively, as demonstrated by virus titers ($TCID_{50}$/ml) determined in cell culture. This indicates that, in general, siRNAs can inhibit BHV-1 multiplication *in vitro* and could be used also against a BHV-1 infection *in vivo* following optimization of their application.

Keywords: siRNAs; Bovine herpesvirus 1; UL25 gene

Introduction

RNA-mediated gene silencing is a highly conserved natural mechanism described for plants, fungi, worms, insects, and mammals. It is mediated by 21 to 25-bp short siRNAs. The siRNAs bind to RNA-induced silencing complex forming an activated complex, which targets mRNA with sequence complementary to the siRNAs. This renders the mRNA transcripts inert and degradable resulting in a specific reduction of the targeted gene expression. The sequence-specific knockdown of viral genes in infected cells generated great interest in development of siRNAs-based antiviral therapeutics. siRNAs-based antiviral activity has been reported for many RNA and DNA viruses *in vitro* and *in vivo* (Ma *et al.*, 2007). The siRNAs-based antiviral activity has been reported against different herpesviruses including Herpes simplex virus 1 (HSV-1) (Bhuyan *et al.*, 2004; Yoon *et al.*, 2004; Palliser *et al.*, 2006; Zhang *et al.*, 2007; Muylaert and Elias, 2007; Zhe *et al.*, 2008), Human cytomegalovirus (Wiebusch *et al.*, 2004), Anatid herpesvirus 1 (AnHV-1) (Mallanna *et al.*, 2006) and Pseudorabies virus (Klupp *et al.*, 2006). These studies targeted genes primarily involved in viral DNA synthesis, replication, assembly, and structural genes.

BoHV-1 (the genus *Varicellovirus*, the family *Herpesviridae*) is a major pathogen of cattle causing severe respiratory, reproductive, neonatal, and dermal disease. BoHV-1 can predispose animals, presumably through immunosuppression, to secondary bacterial infections that lead to a severe pneumonia and death (Yates, 1982). After BoHV-1 infection of host cells, the viral nucleocapsid enters into the cytoplasm and genomic DNA translocates into the nucleus. Like with other herpesviruses, BoHV-1 genomic DNA replication and expression of viral proteins leads to an assembly and release of the new virions. In HSV-1 replication, the viral genes namely UL6, UL15, UL17, UL25, UL28, UL32, and UL33 are required for replication, cleavage, and packaging of viral DNA (Addison *et al.*, 1990; al-Kobaisi *et al.*, 1991; Poon and Roizman, 1993; Tengelsen *et al.*, 1993; Lamberti and Weller, 1996, 1998; McNab *et al.*, 1998; Taus *et al.*, 1998). HSV-1 mutated in one of these genes failed to cleave and package concatemeric DNA into capsids (McNab *et al.*, 1998; Stow, 2001).

Since the detection of UL25 gene ORF in BoHV-1 genome homologous to HSV-1 (Desloges *et al.*, 2001), its role

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Abbreviations: BoHV-1 = Bovine herpesvirus 1; siRNAs = small interfering RNAs

Table 1. Template DNA oligonucleotides used in synthesis of siRNAs *in vitro*

Name	Sequence
UL25-siRNA-1F	5'-GGC GCT TGA GTC AGT TTA ACG CCT GTC TC-3'
UL25-siRNA-1R	5'-TTA AAC TGA CTC AAG CGC CGC CCT GTC TC-3'
UL25-siRNA-2F	5'-GCG ACT ACG ACT TGC TGT ACT TT CCT GTC TC-3'
UL25-siRNA-2R	5'-AGT ACA GCA AGT CGT AGT CGC TG CCT GTC TC-3'

The nucleotides complementary to the T7 promoter primer are underlined.

in BoHV-1 replication has been investigated (Desloges and Simard, 2003). Although, the mutant BoHV-1 with UL25 deletion synthesized late viral proteins and viral DNA, but it failed to produce virus progeny indicating that the UL25 protein was essential for the replication of BoHV-1 (Desloges and Simard, 2003). Similarly, the UL25 protein was found necessary for HSV-1 replication (Ali *et al.*, 1996), stable encapsidation (McNab *et al.*, 1998) and viral DNA packaging (Stow, 2001). Further, the role of UL25 in Pseudorabies virus replication was found to be essential (Klupp *et al.*, 2006). The studies on UL25 mutant of BoHV-1, HSV-1, and Pseudorabies virus indicated that UL25 gene silencing could be an effective approach to inhibit BoHV-1 infection.

The aim of the present study was to determine the inhibition effect of exogenously delivered siRNAs targeting UL25 gene on the BoHV-1 multiplication in the cell culture.

Materials and Methods

Virus and cells. The BoHV-1 isolate 216 II characterized as a respiratory isolate of BoHV-1 was used in this study (Gupta and Rai, 1993). Madin-Darby bovine kidney (MDBK) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Invitrogen) and 50 µg/ml gentamicin at 37°C under 5% CO_2.

Design and in vitro synthesis of siRNAs. Two sequences targeting the BoHV-1 UL25 gene (GenBank Acc. No. AJ004801) were chosen: 5'-GGCGCUUGAGUCAGUUUAACG-3' (nt 1706–1726) and 5'-GCGACUACGACUUGCUGUACUUU-3' (nt 1733–1755) for siRNA-1 and siRNA-2, respectively. The sense and antisense template DNA oligonucleotides for the synthesis of siRNAs were chemically synthesized (Table 1) and used for *in vitro* transcription of siRNAs using the Silencer™ siRNA Construction Kit (Ambion). Briefly, the template DNA oligonucleotides were annealed with T7 promoter primers and transcribed with T7 RNA polymerase. The obtained sense and antisense siRNA strands were annealed, digested with both RNase and DNase, purified, and analyzed by PAGE.

Transfection of cells with siRNAs. MDBK cells were transfected with siRNAs using siPORTAmine™ (Ambion) transfection agent following manufacturer's instructions. The 70%–80% confluent MDBK monolayer was transfected with 100 nmol of different siRNAs complex. After 6 hrs post transfection, the siRNAs complex was removed and cells were incubated further for 18–21 hrs before analysis for siRNA effect.

Titration of infectious virus. The MDBK cells transfected with different siRNAs were infected with BoHV-1 at the multiplicity of infection (MOI = 1) and incubated further for 48 hrs. Control cells were infected with MOI = 1 of BoHV-1. After incubation, the cell culture supernatant was harvested and titration of infectious virus was assayed by estimation of $TCID_{50}$/ml following method described earlier (Reed and Muench, 1938).

Results and Discussion

In order to determine whether RNA-mediated gene silencing can inhibit BoHV-1 multiplication in MDBK cells *in vitro*, two siRNAs (siRNA-1 and siRNA-2) targeting the UL25 gene ORF were designed and analyzed. The target sequences were selected within the conserved region among various BoHV-1 strains and by avoiding the potential secondary structure of viral mRNA for UL25 gene. The selected siRNAs were having the least sequence homology with cow genome database to ensure potential antiviral therapeutic application. The concentrations of *in vitro* synthesized siRNAs were 201.6 and 306.9 ng/µl, respectively (Fig. 1). The initial standardization and optimization of transfection experiments was carried out using different concentration of siRNAs (25–200 nmol) in BoHV-1-infected MDBK cells (MOI = 1) to ascertain their relative inhibitory effect. A concentration of 100 nmol of siRNAs showed maximum inhibition and was selected for all subsequent experiments. There was 0.43 $\log_{10}$ (10.9%) and 1.55 $\log_{10}$ (39.5%) reduction in BoHV-1 titer in supernatant collected 48 hrs post infection in MDBK cells transfected with siRNA-1 and siRNA-2, respectively (Fig. 2). Similar reductions in plaque count and plaque sizes were seen with both siRNAs (data not shown). This clearly indicated that both the siRNAs were effective in inhibiting the virus multiplication. Further, the effect was strong with siRNA-2 as demonstrated by reduction in virus titer ($TCID_{50}$/ml) compared to control. The result of this study and results of Desloges and Simard, 2003 with deletion mutant of BoHV-1 indicated that the UL25 protein is essential for the replicative cycle of BoHV-1.

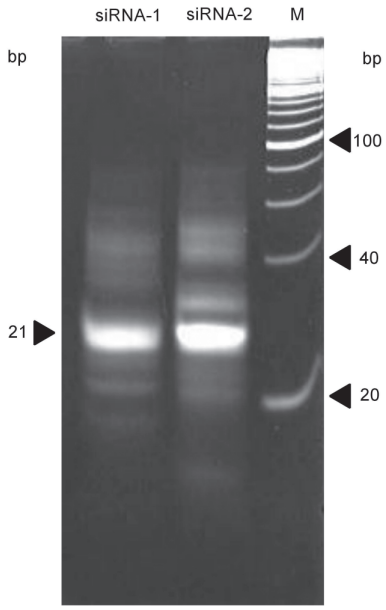

Fig. 1

PAGE of siRNAs

DNA ladder (lane M).

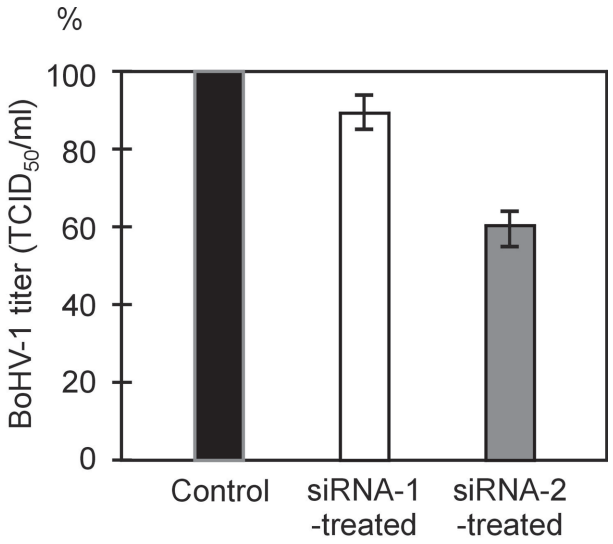

Fig. 2

Inhibition of BoHV-1 multiplication by siRNAs

Although the two siRNAs designed and analyzed in this study had sequences homologous to the BoHV-1 UL25 mRNA, they differed in their efficacy for establishing anti-BoHV-1 activity. In four independent experiments, siRNA-2 consistently inhibited BoHV-1 multiplication more efficiently than siRNA-1 indicating that the difference in efficacy cannot be ascribed solely to differences in transfection efficiency. Variation in the silencing activity of several siRNAs targeting the same mRNA has also been reported previously (Khvorova *et al.*, 2003). The difference in siRNAs efficacy has been attributed to the differences in thermodynamic properties of different siRNAs. Further, the secondary and tertiary structure at the RNA target site also plays an important role in determining the efficacy of the siRNAs (Khvorova *et al.*, 2003).

In conclusion, the present study showed that BoHV-1 was susceptible to the siRNAs targeting UL25 gene. Both tested siRNAs resulted in the inhibition of BoHV-1 multiplication, reduction of virus titer, and in a change of number and size of virus plaques. These results implicate the usefulness of siRNA therapy against BoHV-1 infection, although the delivery of siRNAs *in vivo* has to be carefully optimized.

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