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Therapeutic application of RNA interference against foot-and-mouth disease virus *in vitro* and *in vivo*

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ABSTRACT

Foot-and-mouth disease (FMD) is an economically important animal disease because of the speed of its transmission. Routine vaccination may not be effective for early protection in an outbreak situation. Small interfering RNA (siRNA) can be used in a rapid and effective antiviral approach. However, siRNA has limitations when used in disease prevention, such as a short duration of action. In this study, we have demonstrated that treatment with siRNA after FMD virus (FMDV) infection has an antiviral effect and could be effective in control of FMDV. We applied adenoviruses expressing siRNA both before and after FMDV infection *in vitro* and *in vivo*. Treatment after FMDV infection gave effective viral inhibition, but a combination of treatment before and after FMDV infection gave the best results in IBRS-2 cells. We obtained high survival rates in suckling mice by the use of therapeutic injections following challenge. The results of this study suggest that treatment with siRNA could enhance antiviral effects and may be helpful in the control of FMDV in an outbreak.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease that affects cloven-hoofed animals such as cattle, swine and sheep (Pereira, 1981). Because FMD virus (FMDV) can spread rapidly between susceptible animals it is listed by the World Organisation for Animal Health (OIE) and is, politically and economically, one of the most important animal diseases in the world. FMDV belongs to the genes *Aphthovirus* in the family *Picornavirudae* (Bachrach, 1968; Domingo et al., 2003). The virus genome comprises 8.5 kb of positive-sense single-stranded RNA containing one open reading frame. There are seven serotypes of FMDV: A, O, C, Asia1, SAT1, SAT2 and SAT3, but numerous subtypes have evolved within each serotype (Knowles and Samuel, 2003).

The phenomenon of RNA interference (RNAi) is mediated by short hairpin dsRNA (shRNA), and causes gene silencing in a sequence-specific manner. The inhibition of virus replication by RNA interference has been reported for several viruses associated with human disease, including poliovirus, human immunodeficiency virus-1 (HIV-1) and hepatitis virus (Jacque et al., 2002; Hamasaki et al., 2003; Lee and Rossi, 2004; Gitlin et al., 2005; Saulnier et al., 2006). Recently, inhibition of FMDV replication using RNA interference has been reported. Kahana et al. demonstrated that RNAi could decrease the replication of FMDV in BHK-21 cells (Kahana et al., 2004). A plasmid expressing shRNA targeting VP1 region (Chen et al., 2004) and adenoviruses expressing shRNA targeting 1D and 3D region (Chen et al., 2006) have been shown to possess antiviral activity against FMDV *in vitro* and *in vivo*.

The use of current FMD vaccines to induce early protection is limited because complete protection cannot induce until 7 days, and alternative methods to reduce the spread of FMDV rapidly in outbreak situations are needed. The application of RNAi is one of the possible alternative strategies for FMDV control because it is a rapid and effective antiviral approach. However, the short duration of effect of RNAi has been identified as a challenge that currently limits the use of RNAi as an effective antiviral agent (Bayry and Tough, 2005).

In this study, the antiviral effect of small interfering RNAs (siRNAs) on virus replication both before and after viral infection was investigated. We have demonstrated that treatment using RNA interference after virus infection enhances the antiviral effect and may be a promising alternative strategy for FMDV control.

Abbreviations: FMDV, foot-and-mouth disease virus; siRNA, small interference RNA; shRNA, short hairpin RNA; RNAi, RNA interference; ELISA, enzyme-linked immunosorvent assay; RT-PCR, reverse transcriptase polymerase chain reaction; $TCID_{50}$, 50% tissue culture infective dose; dpc, days post-challenge; p.i., post-infection; LD_{50}, 50% lethal dose.

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2. Materials and methods

2.1. Cells, animals, viruses and virus titration

Human embryonic kidney cells, including human adenovirus type 5 E1 DNA (293 A cells), and swine kidney cells (IBRS-2) were propagated in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS; pH 7.4) at 37 °C with 5% CO_2. CD-1 (ICR) suckling mice, 7 days old and weighing 4–5 g, were purchased from Orient Co. Ltd. (South Korea). Animals were treated in accordance with the ethical guidelines of the animal welfare committee of National Veterinary Research and Quarantine Service (NVRQS). FMDV isolates of strain O/SKR/2002 were used for viral challenge.

Virus titers were determined on IBRS-2 cells for FMDV and 293A cells for adenovirus. The 50% tissue culture infective dose ($TCID_{50}$) was calculated using the formula of Reed and Muench (1938).

2.2. Design and construction of plasmid

The siRNAs were developed from FMDV O/SKR/2000 (GenBank accession no. AJ539139) and O/SKR/2002 (GenBank accession nos. AY312589 and AY312588) strain sequences using Blast and Turbo si-Designer Program (Bioneer Corporation, South Korea). The target sequences of the siRNAs are summarized in Table 1. The siRNA of housekeeping gene Lamin A/C was used as a negative control because it lacks any sequence homology with FMDV. Oligonucleotides of the inverted repeat FMDV target sequence were annealed and cloned into the pENTR™/U6 entry vector (Invitrogen, SanDiego, CA, USA) under control of the U6 promoter and a termination signal consisting of six thymidines. To generate a recombinant adenovirus construct expressing the target sequences, we performed recombination reaction with the pENTR™/U6 entry construct and pAd/BLOCK-iT-DEST vector (Invitrogen, SanDiego, CA, USA) using LR clonase II enzyme mix (Invitrogen, SanDiego, CA, USA).

2.3. Adenovirus production

Recombinant human adenovirus was produced following the manufacturer's instructions. Briefly, the recombinant adenovirus constructs were linearized with Pac I and purified by ethanol precipitation. The Pac I digested adenovirus constructs were transfected into 293A cells using Lipofectamine 2000 (Invitrogen, SanDiego, CA, USA). When approximately 80% CPE was observed, the adenoviruses were harvested and amplified. Adenovirus stocks of 10^7 to 10^8 $TCID_{50}$/ml were used for the experiment.

2.4. Ad-short hairpin RNA inoculation and viral challenge in IBRS-2 cells

2.4.1. Pre-treatment with adenovirus

IBRS-2 cells were plated to 96-well plates at a concentration of 3.5×10^4 cells/well. After day, 90% confluence of IBRS-2 cells was present at the time of infection. Cells were inoculated with Ad-shRNA at 3×10^6 $TCID_{50}$. After 12 h (h) absorption, the Ad-shRNA suspension was removed and the cells were washed twice with D-MEM. The IBRS-2 cells were infected immediately with 100 μl (100 $TCID_{50}$/0.1 ml) of FMDV (this was performed 12 h post-inoculation with adenoviruses). After 1 h absorption at 37 °C, the inocula were removed and the cells were washed twice with D-MEM. Two hundred microlitres of culture medium containing 2% FBS was added and the cells were incubated at 37 °C. Supernatants were collected at 24 h, 36 h and 48 h following FMDV infection and were used in the kinetic analysis of FMDV.

2.4.2. Pre- and post-treatment with adenovirus

Ad-shRNA, adenovirus mixture of Ad-3C and Ad-2B (Ad-3C + 2B) at 3×10^6 $TCID_{50}$ was added to IBRS cells and incubated at 37 °C to detect the effect of post-treatment with adenovirus. After adsorption for 2 h, the inocula were removed and the cells were washed twice with D-MEM. One hundred microliters of culture medium containing 2% FBS was added and the cells were incubated at 37 °C. IBRS-2 cells were infected with 100 μl (100 $TCID_{50}$/0.1 ml) of FMDV 6 h following inoculation with adenovirus. After 1 h absorption at 37 °C, the inocula were removed; Ad-shRNA, Ad-3C + 2B was added at 3×10^6 $TCID_{50}$ and the cells were incubated at 37 °C for 2 h. The inocula were then removed and the cells were washed twice with D-MEM. Following this, 200 μl of culture medium containing 2% FBS was added and the cells were incubated at 37 °C. Supernatant was collected at various times (24 h, 48 h and 72 h p.i.) following FMDV infection and used for evaluation of FMDV replication.

2.4.3. Post-treatment with adenoviruses at various time intervals

Ad-shRNA, adenovirus mixture of Ad-3C and Ad-2B (Ad-3C + 2B) was added to IBRS-2 cells at 3×10^6 $TCID_{50}$ and incubated at 37 °C to detect the effect of post-treatment with adenovirus-mediated RNAi. After 12 h absorption, Ad-3C + 2B suspension was removed and the cells were washed twice with D-MEM. Cells were then infected immediately with 100 μl (100 $TCID_{50}$/0.1 ml) of FMDV. After 1 h absorption at 37 °C, the inocula were removed and the cells were washed twice with D-MEM. Treatment with adenoviruses expressing shRNA was carried out at various time intervals following FMDV infection (1 h, 12 h, or 24 h p.i.). Culture medium was added to give a volume of 200 μl in all wells and the cells were incubated at 37 °C. Supernatant was collected at various time intervals following FMDV infection (24 h, 48 h and 72 h p.i.). Ad-Lamin A/C was treated as above at 12 h before and (or) 1 h after FMDV infection.

2.5. Analysis of FMDV replication in IBRS-2 cells

To assay the effect of adenovirus-mediated shRNA on FMDV RNA replication, quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR), FMDV antigen enzyme-linked immunosorbent assay (ELISA) and virus titrations were carried out. The copy numbers of FMDV RNA in the supernatant

Table 1
Target sequences of shRNAs used in this study

Name	Target sequence (nucleotide position of O/SKR/2002)	Oligonucleotide sequence
Ad-3C	5′-GAGTGTTTGAGTTTGAGAT-3′ (nt 6240–6258 in the 3C region)	5′-CACCGGAGTGTTTGAGTTTGAGATCGAATCTCAAACTCAAACACTC-3′ (top strand) 5′-AAAAGAGTGTTTGAGTTTGAGATTTCG TCTCAAACTCAAACACTCC-3′ (bottom strand)
Ad-2B	5′-GCAGGAGGACATGTCAACA-3′ (nt 4012–4030 in the 2B region)	5′-CACCGGCAGGAGGACATGTCAACACGAATGTTGACATGTCCTCCTGC-3′ (top strand) 5′-AAAAGCAGGAGGACATGTCAACATTCGATGTTGACATGTCCTCCTGCC-3′ (bottom strand)
Ad-Lamin A/C	5′-CTGGACTTCCAGAAGAACA-3′	5′-CACCGCTGGACTTCCAGAAGAACACGAATGTTCTTCTGGAAGTCCAG-3′ (top strand) 5′-AAAACTGGACTTCCAGAAGAACATTCGTGTTCTTCTGGAAGTCCAGC-3′ (bottom strand)

Sense and antisense target sequences are underlined.

Table 2
The schedule of injections with adenovirus preparations

Group	Treatment	Time of injection with adenovirus			
		−1 dpc[a]	−6 hpc[b]	0 dpc	3 dpc
Ad-3C	Twice	Injection	Injection	Challenge	–
Ad-2B	Twice	Injection	Injection	Challenge	–
Ad-3C + 2B	Twice	Injection	Injection	Challenge	–
Single treatment (Ad-3C + 2B)	Once	–	Injection	Challenge	
Two treatments (Ad-3C + 2B)	Twice	Injection	Injection	Challenge	–
Three treatments (Ad-3C + 2B)	Three times	Injection	Injection	Challenge	Injection

[a] Days post-challenge.
[b] Hours post-challenge.

was determined by quantitative real-time RT-PCR. Viral RNA was extracted using a BioRobot M48 workstation and Mag-Attract viral RNA kit (Qiagen, Valencia, CA, USA). Real-time RT-PCR was conducted using a Quantitect Probe RT-PCR kit (Qiagen, Valencia, CA, USA). All assays were performed according to the manufacturers' instructions. The primers targeting the 2B region were: sense 5′-AGATGCAGGARGACATGTCAA-3′ and antisense 5′-TTGTACCAGGGYTTGGCYT-3′. The probe was: 5′-mAAACACGGACCCGACTTTAACCGxp-3′. Its 5′end was labeled with 6-FAM whereas the 3′end was labeled with TAMRA (Oem et al., 2005). Amplification and product detection were performed using Light Cycler System 1.0 (Roche Diagnostics, Mannheim, Germany).

The level of FMDV antigen in the cells supernatants was determined by antigen ELISA using a test kit produced by the Pirbright Laboratory, Institute for Animal Health, UK (Roeder and Le Blanc Smith, 1987). The assays were performed according to the manufacturer's instructions. The virus titer ($TCID_{50}$) of each supernatant was determined on IBRS-2 cells. Statistical analysis was performed using the Student's *t*-test and ANOVA in commercial software Graph Pad Instat.

2.6. Viral challenge in suckling mice

To investigate the antiviral effect of adenoviruses expressing shRNA *in vivo*, 7-day-old suckling mice were used. The dose of FMDV was determined in four 10-fold serial dilutions of virus. The 50% lethal dose (LD_{50}) of FMDV was estimated using the Reed–Muench method (Reed and Muench, 1938). Suckling mice were inoculated by intraperitoneal (IP) injection with 1.5×10^7 $TCID_{50}$ of adenovirus preparation. Control mice were inoculated with the same titer of Ad-Lamin A/C. After 6 or 24 h, the suckling mice were challenged with $25LD_{50}$ or $125LD_{50}$ of FMDV (O/SKR/2002) in 0.05 ml by IP injection (Table 2). Post-treatment with adenovirus was conducted at 2 days post-challenge (dpc) at the same dose as that used for pre-treatment. The animals were monitored for 7 days. Statistical analysis was performed using the log-rank test in commercial software GraphPad Prism (Version 4.0).

3. Results

3.1. Inhibition of FMDV replication in IBRS-2 cells by single or dual treatment with adenovirus-mediated shRNAi

The level of FMDV RNA measured using real-time quantitative RT-PCR was reduced in cells infected with adenovirus-mediated shRNAs (Fig. 1A). In particular, Ad-3C was remarkably effective, and the mixed treatments containing adenoviruses were more effective than Ad-2B. Ad-3C and a mixture of Ad-2B and Ad-3C showed more than 90% viral inhibition at 48 h post-infection. Ad-2B also displayed significant levels of inhibition (approximately 70%).

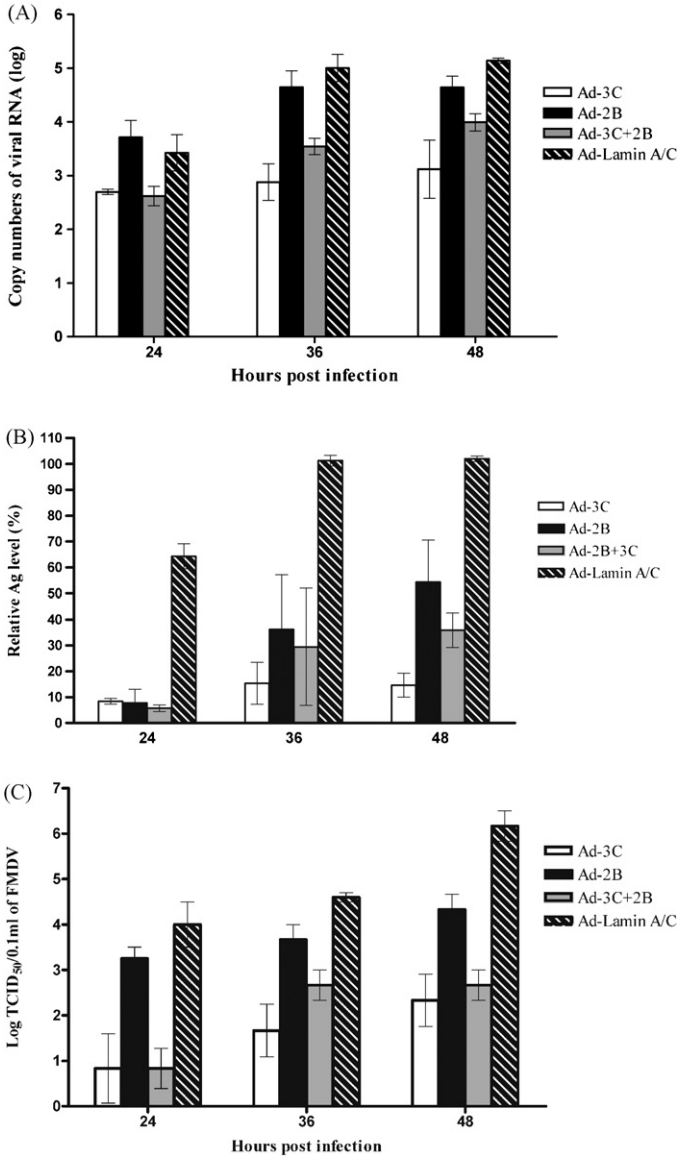

Fig. 1. Adenoviruses expressing siRNAs effectively inhibited FMDV replication in IBRS-2 cells. Cells were inoculated with Ad-shRNA at 3×10^6 $TCID_{50}$. Recombinant adenovirus targeting the Lamin A/C gene used as a negative control. After 12 h, IBRS-2 cells were infected with 100 $TCID_{50}$ of FMDV O/SKR/2002. Samples of supernatant were taken at 24 h, 36 h and 48 h following FMDV infection. The amount of FMDV RNA was determined by quantitative real-time RT-PCR. Total RNA was extracted from the supernatant and used for quantitative real-time RT-PCR (A). FMDV protein levels were determined by antigen ELISA. Assays were performed according to manufacturer's instructions (B). The titer of FMDV was determined by $TCID_{50}$ in IBRS-2 cells (C). Error bars indicate standard deviation (S.D.).

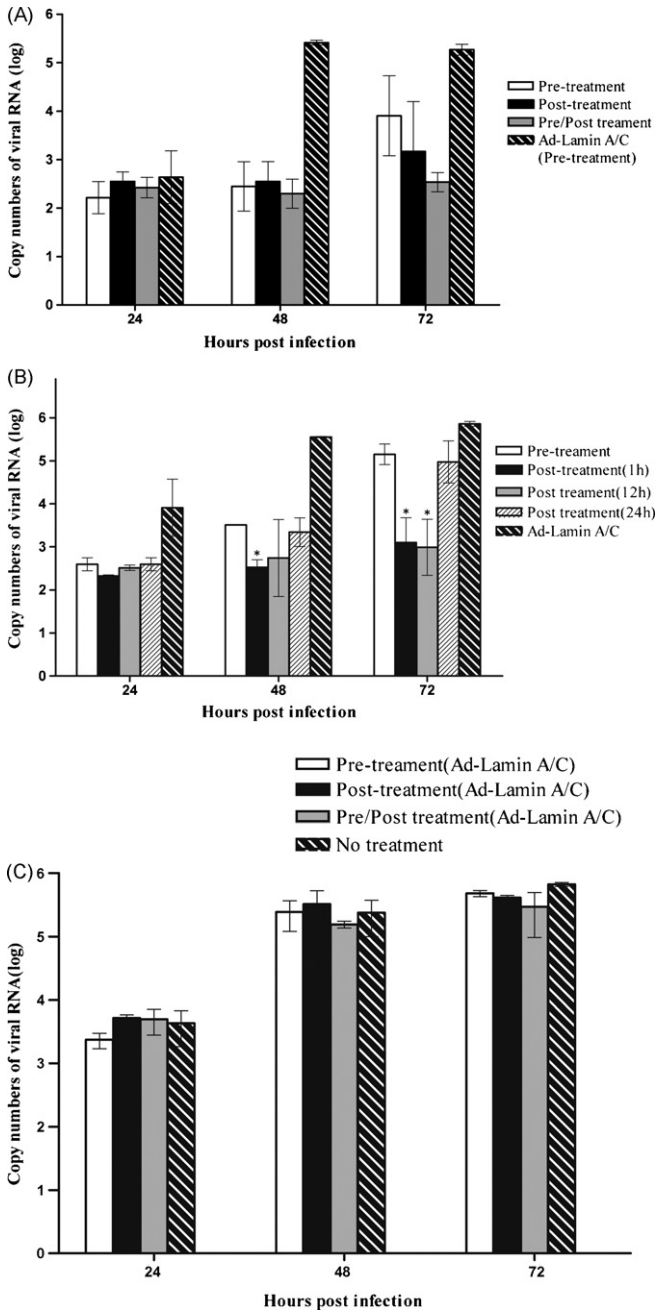

Fig. 2. Post-treatment with Ad-shRNA, with adenovirus mixture of Ad-3C and Ad-2B (Ad-3C + 2B) effectively enhanced FMDV replication in IBRS-2 cells. (A) Cells were inoculated with Ad-3C + 2B at 3×10^6 $TCID_{50}$ for 2 h in pre-treatment and pre/post-treatment but not post-treatment groups. IBRS-2 cells were infected with 100 $TCID_{50}$ of FMDV O/SKR/2002, 6 h following inoculation of adenoviruses. After 1 h, the supernatant was removed and cells were then inoculated with Ad-3C + 2B at 3×10^6 $TCID_{50}$ for 2 h in post-treatment and pre/post-treatment but not pre-treatment groups. (B) Cells were inoculated with Ad-3C + 2B at 3×10^6 $TCID_{50}$ for 2 h. IBRS-2 cells were infected with 100 $TCID_{50}$ of FMDV O/SKR/2002, 12 h following inoculations of adenoviruses. After 1 h absorption, the inocula were removed and cells were washed twice with D-MEM. After various time intervals (1 h, 12 h or 24 h) following FMDV challenge, cells were inoculated with Ad-3C + 2B at 3×10^6 $TCID_{50}$. A statistically significant difference in copy number was observed between pre-treatment and 1 h and 12 h post-treatment groups at 72 h p.i. (*$P < 0.05$, Student's *t*-test). (C) Cells were inoculated with Ad-Lamin A/C at 3×10^6 $TCID_{50}$ as (B) except no treatment group. After 1 h interval following FMDV challenge, cells were inoculated with Ad-Lamin A/C in post and pre/post group. All values are statistically not significant ($P > 0.05$, ANOVA). In both (A) and (B), recombinant adenovirus targeting Lamin A/C gene was used as a negative control. Samples of supernatant were taken at 24 h, 48 h and 72 h following FMDV infection. The amount of FMDV RNA was determined by quantitative real-time RT-PCR. Total RNA was extracted from the supernatant and used for quantitative real-time RT-PCR. Error bars indicate standard deviation (S.D.).

The relative viral protein level was measured at 24 h, 36 h and 48 h p.i. using antigen ELISA. The results show that the relative level (%) of FMD viral protein was reduced in IBRS-2 cells infected with adenovirus-mediated shRNAs (Fig. 1B). This finding was consistent with the results of the real-time quantitative RT-PCR.

To further demonstrate the effect of Ad-mediate shRNAs on FMD viral replication, we determined the virus titers ($TCID_{50}$) of the cell supernatants (Fig. 1C).

3.2. Enhanced antiviral effect in IBRS-2 cells by post-treatment with shRNA

To determine if the timing of treatment with shRNA could influence the antiviral effect against FMDV in cells, we inoculated recombinant adenoviruses into IBRS-2 cells. To demonstrate the degree of inhibition of viral RNA replication, viral RNA was extracted from the supernatant and real-time quantitative RT-PCR was performed (Fig. 2A). At 24 h, the pre-treatment group had the lowest copy number value but, the difference in copy number among all groups were not statistically significant ($P > 0.05$, ANOVA). At 48 h there was a significant reduction in copy number for the pre-, pre/post- and post-infection samples compared to the Ad-Lamin A/C control ($P < 0.001$, *t*-test), but the no difference between these three. At 72 h, the copy number was increasing in all but the pre/post sample, which maintains its markedly lower copy number. An enhanced antiviral effect with post-treatment was observed at 72 p.i.

To investigate the optimal timing of treatment with adenovirus-mediated shRNA, we inoculated recombinant adenoviruses into IBRS-2 cells at various time intervals following infection (1 h, 12 h and 24 h) (Fig. 2B). Cells were treated with adenovirus prior to FMDV infection or prior to and after FMDV infection. 1 h or 12 h post-treatment group showed greater antiviral effect at 48 and 72 h p.i. than pre-treatment and 24 h post-treatment group. A statistically significant difference in copy number was observed between the pre-treatment group and the 1 h ($P = 0.044$, *t*-test) and 12 h post-treatment ($P = 0.048$, *t*-test) groups at 72 h p.i. However, the difference in copy number between the pre-treatment and 24 h post-treatment groups was not statistically significant at 72 h p.i. At 48 h p.i., the group treated 1 h p.i. showed the greatest antiviral effect ($P = 0.02$, *t*-test). Fig. 2B shows that 1 h post-treatment is most effective among post-treatment groups.

To exclude the possibility that the enhanced post-treatment effects are due to a non-specific consequence of challenging an FMDV infected cell with recombinant adenovirus, we inoculated adenovirus control, Ad-Lamin A/C into IBRS-2 cells at 12 h before and (or) 1 h after FMDV infection (Fig. 2C). The difference in copy number among all groups were not statistically significant at 24 h, 48 h and 72 h p.i. ($P = 0.24$, 0.87 and 0.16, ANOVA). It was demonstrated that the inhibition effect of post-treatment with adenovirus-mediated shRNA was not due to a non-specific consequence and the Ad-Lamin A/C was suitable for a negative control in this study.

3.3. Antiviral effects of adenovirus-mediated RNAi in suckling mice

To test the anti-FMDV effect of adenovirus-mediated shRNAs *in vivo*, we challenged suckling mice that had been pre-treated with adenoviruses by IP injection with FMDV O/SKR/2002. Control mice,

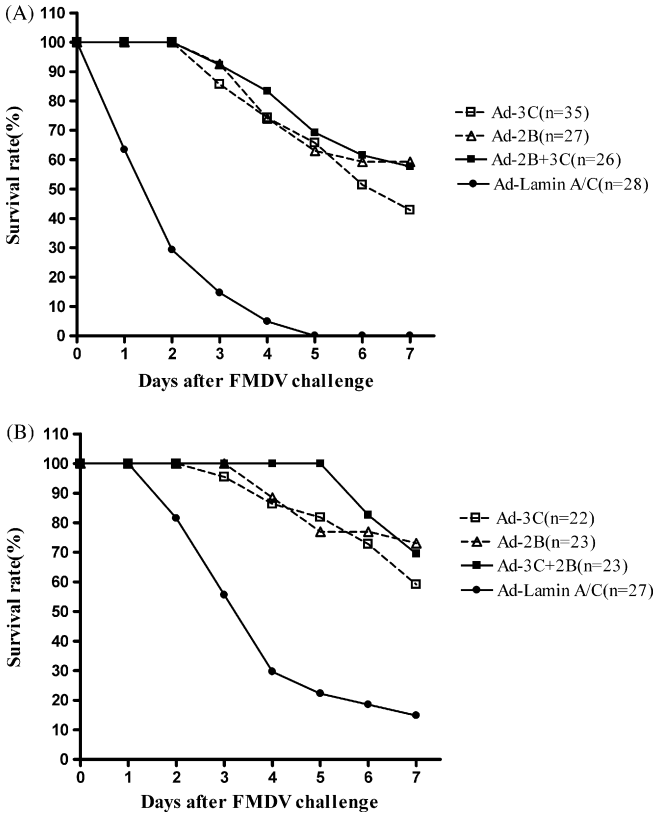

Fig. 3. Survival rates in suckling mice after FMDV challenge were enhanced by treatment with recombinant adenovirus. Suckling mice were treated with adenoviruses targeting the 3C or 2B region or an adenovirus mixture of Ad-3C and Ad-2B. After 6 h and 24 h of adenovirus treatment, suckling mice were challenged with 125 LD_{50} (A) or 25 LD_{50} (B) of O/SKR/2002 by intraperitoneal injection. Animals were monitored for 7 days.

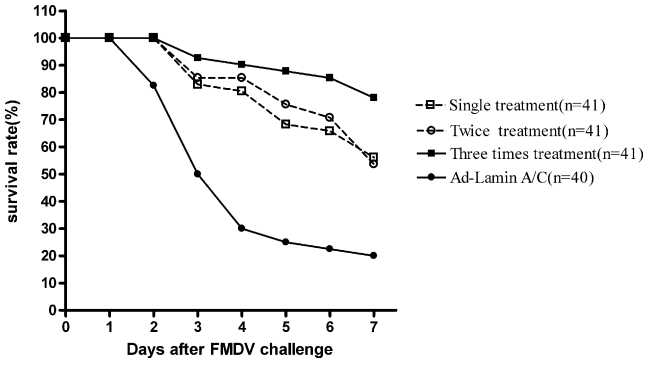

Fig. 4. Post-treatment with adenoviruses enhanced survival rates after FMDV challenge. Suckling mice were treated with an adenovirus mixture of Ad-3C and Ad-2B at 1.5×10^6 $TCID_{50}$ each by single injection (6 h before challenge), two injections (6 h and 24 h before challenge) or three injections (6 h and 24 h before challenge and 3 days post-challenge). Suckling mice were challenged with 25 LD_{50} of FMDV (O/SKR/2002).

which were challenged with 125LD_{50} FMDV had a survival rate of approximately 20% at 3 dpc and died within 6 dpc. Treatment with the adenovirus expressing shRNA targeting non-structural protein (NSP) region 2B and 3C inhibited FMDV viral replication in suckling mice ($P < 0.0001$, log-rank test) (Fig. 3A). The results obtained with a mixture of recombinant adenoviruses were similar to the results obtained with treatment with a single adenovirus. The survival rate in treated mice was approximately 58% at 7 dpc. Mice challenged with 25 LD_{50} FMDV had higher survival rates. The difference between the survival rates of mice treated with Ad Lamin-A/C and Ad 2B, 3C, or a mixture was statistically significant ($P < 0.0001$, log-rank test) (Fig. 3B). Treatment with a mixture of recombinant adenoviruses slightly enhanced the survival rate of suckling mice compared with single treatments until 6 dpc. However, the survival rate of mice given the mixture treatment was not significantly higher than that of those given Ad 2B or Ad 3C ($P = 0.77$ and 0.35, log-rank test). Furthermore, an additional injection of adenovirus at 3 dpc enhanced the survival rate following FMDV challenge (Fig. 4). A statistically significant difference in mouse survival was seen between the group treated three times and those given only one or two treatments ($P = 0.0007$ and 0.014) log-rank test. The group treated twice showed a slightly enhanced survival rate compared with the group given a single treatment; however, the difference was not statistically significant ($P = 0.23$, log-rank test). Therefore, treatment with adenovirus-mediated shRNA post-challenge was shown to efficiently enhance the survival rates following FMDV infection.

4. Discussion

The use of siRNAs corresponding to FMDV genes can be a powerful tool for inhibition of virus replication and may be a potential alternative strategy for FMDV control. We have shown that recombinant adenoviruses expressing 2B or 3C shRNA had antiviral effects against FMDV in IBRS-2 cells and suckling mice. However, we acknowledge the short duration of effect of RNAi and also investigated the therapeutic application of siRNA against FMDV.

Specific gene silencing induced by dsRNA injection in one region has been shown to spread to many tissues in *Caenorhabditis elegans*, and systemic RNA interference defective-1 (SID-1), a transmembrane protein that mediates a systemic RNAi effect, has been identified in *C. elegans*, humans and mice (Fire, 1999; Winston et al., 2002; Feinberg and Hunter, 2003). However, siRNA has a similarly short duration of effect and cannot induce the critical level of immunity required for complete viral clearance in mammalian cells (Bayry and Tough, 2005). Chen et al. demonstrated an antiviral effect that lasted only 5 days post-challenge in suckling mice following transfection of an shRNA plasmid targeting VP1 (Chen et al., 2004). Song et al. identified that HIV suppression mediated by viral P24 siRNA was lost by day 7 post-transfection in human monocytes (Song et al., 2003). These results suggest that degradation of siRNA may contribute to the loss of siRNA activity. In another study, viral P24 siRNA transfected after HIV infection was able to suppress viral replication throughout a 15-day period; in contrast, P24 siRNA persisted for only up to 7 days after transfection in uninfected cells (Song et al., 2003). These results suggest that the continued presence of target RNA may encourage persistence of siRNA.

We used three strategies to attempt to enhance the antiviral effect of siRNA and to overcome its short duration of action *in vivo*. First, we attempted to improve the antiviral effect by use of a mixture of two adenovirus constructs in challenge experiments. This strategy was also intended to defend against the high genetic variability of the virus and the production of viral escape mutants (Wilson and Richardson, 2005). We selected regions highly conserved among serotypes for the siRNA target sequences for the same reason. RNA interference targeting the 3C region significantly inhibited FMDV replication, and a mixture of shRNAs targeting the 2B and 3C regions was less effective than that targeting the single 3C region *in vitro*. Surprisingly, there was no synergy between the different siRNAs in IBRS-2 cells. However, a mixture of shRNAs targeting the 2B and 3C regions was more effective than the shRNA targeting 3C *in vivo*. The survival rate of the group treated with the mixture was significantly increased following challenge with 25LD_{50}, and slightly

increased following challenge with 125LD_{50}. Vector-based short hairpin RNAs are processed to siRNAs by dicer-dependent cleavage in the cytoplasm, and then each siRNA is incorporated into an RNA-induced silencing complex (RISC). Toshiyasu et al. reported that the gene silencing effect of RNAi depended on Dicer, enzyme from the RNase III family (Mikuma et al., 2004). The effect of RNAi can depend on cell specificity or the limited quantity of Dicer present in cell lines. It can be predicted that using multiple siRNAs may lead to saturation of RISC. Another possibility is that a mixture of siRNAs may have an advantage in providing a long-term silencing effect. Viruses may produce more mutants able to escape siRNA over a longer period, and we observed virus replication for 7 days *in vivo* and 2 or 3 days *in vitro*. However, the survival rates of the mixture group also declined to the level of those given the single construct at 6 dpc following challenge with 25LD_{50}.

Secondly, we tried to improve the antiviral effect by giving two injections of shRNA prior to FMDV challenge. We tested the antiviral effect in the groups challenged with 25LD_{50} because challenge with a low titer of FMDV was adequate to investigate differences in the antiviral effect among groups. In a previous study, Chen et al. observed that giving a second treatment before challenge does not increase protection in guinea pigs (Chen et al., 2006). We also demonstrated that the difference in survival rate between groups treated once or twice before challenge was not statistically significant. Furthermore, the survival rate of the group given two injections was lower than that of the single injection group at 7 dpc.

Finally, we tried to improve the antiviral effect by giving injections after FMDV challenge. We maintained the high survival rate by treatment with adenoviruses post-challenge. This showed that post-challenge treatments with siRNA are effective in inhibition of viral replication, in addition to pre-treatment with siRNA, and the longer lasting effect of siRNA could be helpful in enhancing the antiviral effect. The result is in agreement with previous studies in cells: siRNAs have been shown to suppress viral replication effectively for prolonged periods in previously FMDV-infected BHK-21 cells (Liu et al., 2005). The previous study showed that the effect of RNAi could be enhanced by transfection of siRNA 1 h following FMDV infection. Our results also demonstrated the effectiveness of treatment with adenoviruses 1 h following FMDV infection in IBRS-2 cells. Furthermore, the combination of treatment with adenoviruses both before and after challenge with FMDV gave the best result, and the antiviral effects were significantly prolonged by treatment 1 h and 12 h post-infection.

These results could be explained by susceptibility of FMDV to siRNA both before and after infection. Another possible explanation is that the activation of enzymes related to RNA interference may enhance the siRNA effect against FMDV. Several critical enzymes, such as Dicer, RNA-dependent RNA polymerase (RdRP) and other enzymes that generate siRNA, may be up-regulated during viral infection. Therefore, when specific siRNA is introduced into virally infected cells, the antiviral response based on siRNA will be faster, effective and long lasting (Bagasra and Prilliman, 2004).

The usefulness of RNAi for the control of FMD has been discussed previously, and RNAi against FMDV has been applied to one of the natural hosts, pigs (Chen et al., 2006). Chen et al. have suggested that an antiviral strategy based on a combination of vaccination, treatment with an emergency agent, slaughter of infected animals and reduction of contamination on affected farms should be effective (Chen et al., 2005). We propose that small interfering RNA could be effective when used as an emergency agent for suspected cases, including persistently infected or susceptible animals. Therapeutic application of siRNA could be used to control persistent infection with FMDV. Following FMDV infection, non-structural protein testing cannot differentiate between fully recovered and carrier animals, although the principal aim of post-outbreak surveillance is to detect acute or persistent infection (Paton et al., 2006). To apply effective treatment for persistent infection, the delivery vector could be modified to target the specific tissues of the oesophagopharyngeal region. In addition, the RNAi strategy could be modified to provide a simple injection method for practical application in the field and combined with interferon for enhanced antiviral effect. In vaccine strategy or long-term inhibition, adenovirus delivery system has the limitation that antibody against adenovirus could be induced by repeating injection of recombinant adenovirus and the antibody could decrease the efficacy of delivery. Our strategy was more rapid prevention and treatment against FMDV compared to them. To circumvent the limitation, single injection or reinjection at short intervals could be effective for therapeutic application.

In conclusion, this study demonstrated that treatment using RNA interference was effective following FMDV infection as well as when used as a preventive measure. We used three strategies to overcome short duration of siRNA effect *in vivo*. We attempted to improve the antiviral effect by use of a mixture of two adenovirus constructs, by giving two injections of shRNA prior to FMDV challenge and by giving injections after FMDV challenge. The high survival rate was maintained by treatment with adenoviruses post-challenge. These results could be explained by susceptibility of FMDV to siRNA both before and after infection or the activation of enzymes related to RNA interference. Small interference RNA is effective for the therapeutic application against FMDV and could be used to control persistent infection with FMDV.

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