Adenovirus-Mediated RNA Interference against Foot-and-Mouth Disease Virus Infection both In Vitro and In Vivo

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Foot-and-mouth disease virus (FMDV) infection is responsible for the heavy economic losses in stockbreeding each year. Because of the limited effectiveness of existing vaccines and antiviral drugs, the development of new strategies is needed. RNA interference (RNAi) is an effective means of suppressing virus replication in vitro. Here we demonstrate that treatment with recombinant, replication-defective human adenovirus type 5 (Ad5) expressing short-hairpin RNAs (shRNAs) directed against either structural protein 1D (Ad5-NT21) or polymerase 3D (Ad5-POL) of FMDV totally protects swine IBRS-2 cells from homologous FMDV infection, whereas only Ad5-POL inhibits heterologous FMDV replication. Moreover, delivery of these shRNAs significantly reduces the susceptibility of guinea pigs and swine to FMDV infection. Three of five guinea pigs inoculated with 10^6 PFU of Ad5-POL and challenged 24 h later with 50 50% infectious doses (ID_{50}) of homologous virus were protected from the major clinical manifestation of disease: the appearance of vesicles on the feet. Two of three swine inoculated with an Ad5-NT21–Ad5-POL mixture containing 2×10^9 PFU each and challenged 24 h later with 100 ID_{50} of homologous virus were protected from the major clinical disease, but treatment with a higher dose of adenovirus mixture cannot promote protection of animals. The inhibition was rapid and specific because treatment with a control adenovirus construct (Ad5-LacZ) expressing *Escherichia coli* galactosidase-specific shRNA showed no marked antiviral activity. Our data highlight the in vivo potential of RNAi technology in the case of FMD.

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease affecting more than 33 species of cloven-hoofed animals, including domestic animals such as cattle and swine, and wild animals such as buffalo and goats (30). The disease has been designated by the Office International des Epizooties, World Organization of Animal Health, as a serious disease that spreads rapidly and requires socioeconomic consideration. FMD outbreaks exert a heavy economic toll due to loss of productivity, decline in product quality, the slaughter of millions of infected and in-contact susceptible animals, and trade embargos on animal products (37). The etiological agent of FMD is FMD virus (FMDV), which belongs to the genus *Aphthovirus* of the family *Picornaviridae* (1). The FMDV genome is composed of a positive-sense single-stranded RNA molecule of about 8,500 nucleotides, containing a unique open reading frame. Like other RNA viruses, FMDV is antigenically variable and undergoes rapid mutation. There are seven distinguishable serological types, namely, O, A, C, Asia1, SAT1, SAT2, and SAT3, and more than 65 subtypes.

Current measures for the control of FMD outbreak include routine vaccination, control of animal movement, and slaughter. FMD vaccines based on inactivated virus and adjuvant are effective in eliminating the disease but risk the escape of live virus from animal facilities or from improper vaccine preparations (3, 22). The development of a recombinant peptide vaccine (23) and a synthetic peptide vaccine (35), both of

which are safe and effective, has been reported. However, current vaccines do not induce a protective response until 7 days postvaccination, and a booster inoculation is usually needed. FMDV has the potential to cause explosive epidemics of the disease because of the low dose of virus required for infection, the large numbers of virus particles excreted, the multiple routes of transmission, and the short incubation period, and signs of disease can appear as early as 2 days postexposure. Thus, current measures might not be sufficient to eradicate the virus if an outbreak threatened to spread widely. This situation has underlined the importance of developing antiviral strategies capable of inducing early protection. In this literature, a high-potency emergency vaccine against FMDV could be effective in preventing disease within 4 to 5 days postvaccination (2). Notably, Chinsangaram et al. (9) have demonstrated that swine inoculated with adenovirus expressing porcine alpha interferon were completely protected when challenged 24 h later with FMDV. Furthermore, Moraes et al. (28) have shown that a combination of adenoviruses expressing porcine alpha interferon and FMDV capsid and 3C proteinase coding regions gave immediate, as well as long-lasting, protection against viral challenge.

The newly discovered RNA interference (RNAi) is a naturally occurring posttranscriptional gene silencing mechanism, which is induced by 19- to 27-nucleotide (nt) small interfering RNA (siRNA) molecules homologous to some region of the target gene (19). It is widely believed that this mechanism is the major antiviral system in plants and insects (16). It is conserved in vertebrates (5, 11) and, although there is no direct evidence that RNAi functions as a natural antiviral defense in mammals, inhibition of mammalian virus replication by means of exper-

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imentally induced RNAi has been reported (11, 16). Because of the high rapidity and specificity of RNAi effect, it could complement and improve the traditional tools available to control important animal pathogens.

Previously, we (7, 24) and other researchers (10, 20, 27) have investigated the inhibitory effect of siRNAs on FMDV replication in vitro. Since the data obtained in cell culture have demonstrated that siRNAs can be effective against FMDV in either a specific manner or a cross-inhibitory manner, the potential of RNAi as an antiviral strategy against FMDV in animal systems is of great interest. Grubman and de los Santos (17) discussed the possibility of using RNAi for the control of FMD and identified several challenges facing the use of RNAi as an antiviral agent. Bayry and Tough (4) have raised other concerns, including the complexity of FMD and problems with siRNA treatment. In a previous study (6), we also reported that much more experimental data is needed in order to be able to evaluate the potential of RNAi for controlling FMD outbreaks and suggested that it might be useful as a co-agent to induce rapid resistance before routine vaccination can evoke protective immunity.

Here we demonstrate that short hairpin RNAs (shRNAs) directed against the structural protein 1D and polymerase 3D of FMDV, delivered by recombinant replication-defective human adenovirus type 5 (rAd5), are capable of inhibiting virus replication in both cultured porcine cells (IBRS-2) and in guinea pigs and swine.

MATERIALS AND METHODS

Animals. Male and female guinea pigs weighing 250 to 300 g and large white swine, 2- to 3-month-old and weighing ca. 40 to 50 kg were used to perform viral challenge. All of the animals were housed in disease-secure isolation facilities in an FMDV-free area, and had no previous FMD contact as confirmed by the absence of detectable anti-FMDV antibodies in their sera.

Cells and viruses. Human kidney cells (AD-293) were used to generate and grow rAd5 and determine virus titers. Baby hamster kidney cells (BHK-21) were used to grow FMDV and determine virus infectivity. Antiviral activity of rAd5 expressing shRNA was assayed in swine kidney cells (IBRS-2). All of the cell lines were cultured in Dulbecco modified Eagle medium supplemented with 10% of heat-inactivated fetal bovine serum (pH 7.4). Cultures were incubated at 37°C with 5% CO₂. FMDV isolates of serotype O (HKN/2002 [GenBank accession AY317098] and CHA/99 [GenBank accession AJ318833]) and one pseudorabies virus (PRV) isolate (Ea [GenBank accession AY318876]) were used for viral challenge.

Construction of plasmids. We used the shRNA-expressing plasmids pNT21, pPOL, and pLacZ. The construction of pNT21 has been described previously (7). Briefly, the mouse U6 promoter (P_{U6}) was chemically synthesized from GenBank sequence data (accession number X06980) and cloned into the NdeI/EcoRI sites of pcDNA3.1B(−) vector (Invitrogen, Groningen, The Netherlands), replacing the human cytomegalovirus immediate-early promoter (P_{CMV}), to generate the parent vector, pU6. Then, inverted repeats targeting the genome of FMDV (Fig. 1A) were subcloned into pU6 at the EcoRI/HindIII sites, under the control of P_{U6} and a termination signal of five thymidines (Ts) (Fig. 1B). Plasmid pNT21 contains an inverted repeat corresponding to nt 16 to 36 of the cDNA of HKN/2002 1D, while plasmid pPOL contains an inverted repeat corresponding to nt 1225 to 1280 of the cDNA of HKN/2002 3D. As a control for nonspecific effects we used plasmid pLacZ containing an inverted repeat corresponding to nt 1353 to 1435 of the β-galactosidase gene of *Escherichia coli*, which has no homology to the HKN/2002 genome, as confirmed by sequence analysis. The sequence of the 21-nt oligonucleotide encoding FMDV 1D-specific shRNA was 5'-GAGTCTGCGGACCCGCTGACT-3' (sense); that of the 56-nt oligonucleotide encoding FMDV 3D-specific shRNA was 5'-GAGGCTATCCTCTCCTTTGCACGCGGTGGGACCACATACAGGAGAAGTTGATCTCCGT-3' (sense), and that of the 83-nt oligonucleotide encoding *E. coli* β-galactosidase-specific shRNA was 5'-GAGTGTGATCATCTGGTCGCTGGGAATGAATCAGGCC

FIG. 1. Schematic diagram of target viral mRNA, shRNA-expressing cassette, Ad5 shuttle vector, and rAd5 DNA. (A) The FMDV genome contains a unique open reading frame. The black arrows underneath indicate the sites targeted by FMDV-specific shRNAs. (B) An inverted repeat corresponding to each of the target sequences in the FMDV genome was inserted under the control of P_{U6} and a transcriptional termination signal of five Ts. The shRNA-expressing cassette was then subcloned into the multiple cloning sites of Ad5 shuttle vector pAdTrack-CMV under the control of P_{CMV} and a poly(A) transcription termination signal (An). As a result, transcription of the shRNA-coding insert could be driven by either P_{U6} or P_{CMV}. The synthesized RNAs should therefore fold back to form two types of shRNAs that are finally processed into the putative siRNAs. (C) The resultant Ad5 shuttle vector was cotransfected with the adenoviral backbone plasmid pAdEasy-1 into *E. coli* by electroporation. The recombinant adenoviral DNAs were generated by homologous recombination.

ACGGCGCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCG-3' (sense).

To get a recombinant adenovirus shuttle vector, we used pAdTrack-CMV (Stratagene, La Jolla, CA) (18). For convenient cloning, we digested and further cloned the SalI-XbaI sequences from pNT21, pPOL, and pLacZ into the pAdTrack-CMV vector at its SalI/XbaI sites (Fig. 1B). The resultant recombinant shuttle vectors, in which the expression of shRNAs could be driven by either P_{U6} or by P_{CMV}, were designated pCMV-NT21, pCMV-POL, and pCMV-LacZ, respectively.

Production of rAd5. rAd5 was generated by the procedure of He et al. (18). The recombinant shuttle vectors were linearized with PmeI and cotransformed by electroporation together with the adenoviral backbone plasmid pAdEasy-1 (Stratagene) into *E. coli* BJ5183. The recombinant adenoviral plasmids were generated by homologous recombination. Positive clones were selected and confirmed by making DNA minipreps and digesting with PacI. The resulting adenoviral plasmids (pAd5-NT21, pAd5-POL, and pAd5-LacZ) were linearized with PacI (Fig. 1C), purified by ethanol precipitation, and transfected into AD-293 packaging cells that had been plated in a 25-cm² flask the previous day. The cells were transfected with 5 μg of linearized plasmid DNA using 20 μl of Lipofectamine reagent 2000 (Invitrogen) and monitored for expression of green fluorescent protein (GFP). To generate high-titer viral stock, adenoviruses Ad5-NT21, Ad5-POL, and Ad5-LacZ were harvested, amplified (18), and purified by CsCl gradient centrifugation (21). Final virus yields were 10¹⁰ to 10¹¹ PFU/ml.

Cell transfection and viral challenge. The growth, isolation, and titration of FMDV and PRV were all conducted using cultured BHK-21 cells, and 50% tissue culture infective doses (TCID₅₀) were calculated using the Reed-Muench formula (32). Viral suspensions of 10⁶ to 10⁷ TCID₅₀/ml were used for the experiments. To assess the capacity of rAd5 to inhibit FMDV infection, 0.3 × 10⁴ IBRS-2 cells, a cell line susceptible to rAd5 infection but not permitting productive replication, were plated in each well of 96-well plates. The following day,

monolayers (ca. 95% confluent) of IBRS-2 cells were incubated with rAd5 at multiplicities of infection (MOI) of 1, 5, and 10 in 0.1 ml of Dulbecco modified Eagle medium without fetal bovine serum. After adsorption for 12 h, 100 TCID₅₀ of FMDV (or PRV) per 0.1 ml were added without removing the rAd5 suspension. Infection was then allowed to proceed also without removing the FMDV (or PRV), and the cells were examined microscopically for GFP expression and cytopathic effects (CPE) (12). Images were collected using an Olympus BH-2 microscope and a Nikon E950 video camera at a magnification of $\times 40$ with an exposure time of 1/8 s. Supernatant fluids were harvested at various time after infection, and virus titers (i.e., TCID₅₀) were determined three times on BHK-21 cells.

Kinetic analysis of FMDV replication in IBRS-2 cells. To detect FMDV replication, total RNA was extracted from IBRS-2 cultures with TRIzol reagent (Gibco-BRL), incubated for 1 h at 37°C with DNase RQ1, and subjected to real-time quantitative reverse transcription-PCR (Q-RT-PCR). In brief, real-time Q-RT-PCR was performed in 96-well plates (Bio-Rad, Hercules, CA) in 20- μ l reaction volumes containing the components of a SYBR RT-PCR Kit (Perfect Real Time; TaKaRa, Kyoto, Japan). The 20- μ l reaction mixture contained 10 μ l of SYBR master mix (2 $\times$), 0.4 μ l of 0.2 μ M forward primer, 0.4 μ l of 0.2 μ M reverse primer, 2.0 μ l of a 1- μ g RNA sample, and 7.2 μ l of water. The cycle program consisted of 50°C for 30 min and 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. Primers for retrotranscription of FMDV 3D mRNA were 5'-GAGGCTATCTCTCTTTGC-3' (sense) and 5'-ACGG AGATCAACTTC-3' (antisense). To confirm specific amplification, melting-curve analysis of the RT-PCR products was performed according to the manufacturer's protocol. Fluorescence was measured after each cycle and displayed graphically with iCycler iQ Real-Time PCR Detection System Software Version 3.0A (Bio-Rad, Hercules, CA). RT-PCR products were cloned into T-vector for sequencing.

Viral challenge in guinea pigs and swine. Serotype O FMDV strain HKN/2002 passaged five times in suckling mice was used to challenge guinea pigs, and the virus passaged three times in swine was used for swine experiments. The viruses were titrated on guinea pigs and swine. The dose of FMDV used was determined by means of four 10-fold serial dilutions of virus (i.e., 10^{-4} , 10^{-5} , 10^{-6} , and 10^{-7}) in phosphate-buffered saline (PBS). Guinea pigs (four groups of six animals each) were inoculated intradermally in the left rear foot with 0.1 ml of serially diluted viruses. The swine (four groups of four each) were inoculated in the neck region by intramuscular injection of each animal with 2 ml of serially diluted virus. All of the animals were then monitored for the major clinical sign of FMD, the appearance of vesicles on the mouth or feet. The 50% animal infective dose (ID₅₀) was estimated by the Reed-Muench method (32).

Guinea pig experiment 1. Guinea pigs in several groups of five each were inoculated by intramuscular injection with 1.0×10^6 (or 1.0×10^7) PFU of purified rAd5 in 0.1 ml of PBS. After 24 h (or 72 h) of inoculation, FMDV challenge was carried out by intradermal injection of each animal with 0.1 ml of guinea pig infectious dose 50 ID₅₀ (or 200 ID₅₀) in the left rear foot, as was done for virus titration.

Guinea pigs experiment 2. In a parallel experiment, animals were treated with an Ad5-NT21-Ad5-POL mixture of 0.5×10^7 PFU each and challenged 24 h later with 50 ID₅₀ of HKN/2002. To test whether two successive inoculations themselves protected animals, some groups were vaccinated intramuscularly, first with the Ad5-NT21-Ad5-POL mixture of 0.5×10^7 PFU each and then 24 h later with the same dose of virus. Thereafter, they were immediately challenged.

Swine experiment. Twelve swine were divided into four groups of three animals each. The animals in each group were cohoused in a separate room. All groups were vaccinated by intramuscular injection in the neck area. Group 1 was a mock control group inoculated with 2 ml of PBS. As a negative control, group 2 was inoculated with 4×10^9 PFU of Ad5-LacZ in 2 ml of PBS. Group 3 was inoculated with an Ad5-NT21-Ad5-POL mixture containing 2×10^9 PFU each in 2 ml of PBS. Group 4 was treated with a high dose of Ad5-NT21-Ad5-POL mixture containing 4×10^9 PFU each in 2 ml of PBS. At 24 h later, all animals were challenged by intramuscular injection with 100 ID₅₀ of HKN/2002 in 2 ml of PBS in the neck area. To avoid overexposure to the challenge virus, animals that developed disease were moved to another room, and then observation proceeded.

After challenge, animals were examined daily for clinical signs of FMD, including an increase in body temperature (above 40°C) and the appearance of vesicles on the mouth and feet. The lesion score was determined at various time points postchallenge by determining the number of digits plus mouth with vesicles for each animal. The observations were terminated on day 14 postchallenge in guinea pigs experiments and on day 21 postchallenge in swine experiments, when the animals were humanely killed.

Serological analysis. Blood and serum samples were collected at days 6, 14, and 21 after challenge in swine experiments. Without being frozen, sera were directly tested for FMDV titers (TCID₅₀) on BHK-21 cells cultured in 96-well plates. To confirm FMDV infection, total RNA was extracted from cell cultures and subjected to real-time Q-RT-PCR as described above.

To assess neutralizing antibody responses in the swine, plaque reduction neutralization assays were performed as described previously (26). Neutralizing titers were reported as the highest serum dilution causing a 50% reduction in the number of HKN/2002 plaque on BHK-21 cells.

The presence of antibodies against viral nonstructural protein 3ABC of FMDV in the sera was detected by using a solid-phase blocking enzyme-linked immunosorbent assay (SPB-ELISA) according to the procedures described by Chenard et al. (8). Briefly, ELISA microtiter test plates were coated overnight at 4°C with recombinant 3ABC antigen expressed in *E. coli* (34). After five washes with PBS containing 0.05% Tween 80 (PBST), each well of the plates was filled with 100 μ l of test or reference serum diluted 1:2 in PBST and incubated at 37°C for 60 min. The serum was then removed, and wells of the plates were washed as described above. After rewashing, 100 μ l of swine anti-3ABC immunoglobulin-horseradish peroxidase conjugate was added, and incubation proceeded at 37°C for 30 min. The conjugate was then removed, wells of the plates were washed again, and 100 μ l of a commercially ready-to-use tetramethyl benzidine chromogen substrate was added to the plates. After incubation at 37°C for 10 min, the reaction was stopped by adding 50 μ l of 2.0 M H₂SO₄ per well, and the optical density (OD) at 450 nm was measured by using an ELISA reader. The sera were considered positive if the OD was reduced by $\geq 30\%$ compared to a standard negative serum corrected for background signal by subtracting the OD of the high positive reference serum.

RESULTS

rAd5 expressing FMDV-specific shRNA completely protects IBRS-2 cells from FMDV infection. To test the antiviral activity of the rAd5 constructs, IBRS-2 cells cultured in 96-well plates were inoculated with FMDV-specific rAd5 at an MOI of 1, 5, or 10. As controls, cells were either not treated or were treated with the same dose of rAd5 expressing *E. coli* β -galactosidase-specific shRNA. Twelve hours later, all of the cells were infected with 100 TCID₅₀ of FMDV HKN/2002, FMDV CHA/99, or PRV Ea. Normal swine kidney IBRS-2 cells are fibroblastic in morphology; they grow as monolayers and have a pronounced tendency to adopt a parallel orientation. Viral infection causes a marked CPE, resulting in complete detachment, rounding up, and disintegration of the cells, as observed by microscopy. When the cells were challenged with homologous FMDV HKN/2002, typical CPEs were seen in the cells of the control groups 24 h postchallenge, whereas the cells treated with FMDV-specific rAd5 (Ad5-NT21 or Ad5-POL) at an MOI of 5 or 10 failed to develop any marked CPE at any time (Fig. 2A). In the group treated with an MOI of 1 of FMDV-specific rAd5, the antiviral effect lasted for up to 3 days, suggesting that inhibition was dose dependent. Cross-inhibition was evident in the face of challenge with the heterologous FMDV CHA/99 in response to treatment with Ad5-POL (Fig. 2B). However, a higher dose of rAd5 was needed to maintain the inhibition (data not shown). None of the rAd5 derivatives conferred resistance to the unrelated PRV Ea (Fig. 2C), indicating that the antiviral effect of Ad5-NT21 and Ad5-POL was FMDV specific.

We investigated the effect of rAd5-mediated RNAi on FMDV replication by measuring the TCID₅₀ of supernatants of cell lysates made at intervals postchallenge. In agreement with the microscopic observations, the supernatant TCID₅₀ for HKN/2002 of the cells infected with Ad5-NT21 or Ad5-POL at an MOI of 5 was reduced by $\sim 100\%$ (Fig. 3A). In the case of challenge with CHA/99, Ad5-POL but not Ad5-NT21 com-

FIG. 2. rAd5 expressing FMDV-specific shRNA confers specific resistance to FMDV in IBRS-2 cells. Cells treated with either Ad5-LacZ or FMDV-specific rAd5 at an MOI of 5 were challenged, 12 h posttreatment, with 100 TCID₅₀ of FMDV HKN/2002 (A), FMDV CHA/99 (B), or PRV Ea (C). At 72 h after challenge, the cells were observed with an Olympus BH-2 microscope, and representative bright-field images (left column) and relative fluorescent-field images (right column) were recorded.

pletely prevented virus multiplication (Fig. 3B); virus formation was barely detectable 36 h after challenge (Fig. 3A and B). Neither FMDV-specific rAd5 nor Ad5-LacZ significantly inhibited the replication of the control virus, PRV Ea (Fig. 3C). To further substantiate the levels of inhibition, cells mock treated or treated with rAd5 at an MOI of 5 and challenged with HKN/2002 were collected 12, 24, and 36 h postchallenge and real-time Q-RT-PCR was performed. The level of FMDV

FIG. 3. rAd5 expressing FMDV-specific shRNA protects IBRS-2 cells from virus infection. Cells were inoculated with shRNA-expressing rAd5 at an MOI of 5 and challenged, 12 h postinoculation, with 100 TCID₅₀ of FMDV HKN/2002 (A), FMDV CHA/99 (B), or PRV Ea (C). Culture supernatants were collected at several times after FMDV challenge, and virus yields were measured by TCID₅₀. The data are means $\pm$ the standard deviation of three separate experiments.

FIG. 4. The level of FMDV 3D transcripts in IBRS-2 cells treated with FMDV-specific rAd5. Cells were inoculated with shRNA-expressing rAd5 at an MOI of 5 and challenged 12 h postinoculation with 100 TCID₅₀ of FMDV HKN/2002. At several hours postchallenge (h.p.c.), total RNA was extracted from cultures and subjected to real-time Q-RT-PCR. (A) One amplification plot of two separate experiments is shown. The y axis represents the PCR baseline-subtracted RFU (relative fluorescence units). Cycle number is displayed on the x axis. (B) Cycle threshold (C_T) values are derived from the amplification profiles shown in panel A.

transcripts, as determined by RT-PCR, was significantly reduced in cells treated with FMDV-specific rAd5 (Fig. 4). Melting-curve analysis confirmed the specific amplification of RT-PCR products (data not shown). The real-time Q-RT-PCR results were very reproducible, based on the cycle threshold (C_T) values in two separate experiments (data not shown). These results indicate that FMDV-specific shRNA-expressing rAd5 can effectively and specifically inhibit infection by the virus in IBRS-2 cells.

FMDV-specific rAd5 constructs inhibit virus infection in guinea pigs. To test the in vivo anti-FMDV potential of RNAi, we challenged guinea pigs pretreated by intramuscular injection of rAd5 constructs with FMDV HKN/2002. All PBS-treated animals developed the major clinical sign of FMD, the appearance of vesicles on the feet, within 48 h of challenge (Table 1), and guinea pigs treated with Ad5-LacZ were not protected at all, although the clinical signs were delayed in some animals, as shown in Table 1. However, three of five guinea pigs pretreated with 10⁶ PFU of Ad5-POL and challenged with 50 ID₅₀ of HKN/2002 developed none of the clinical signs such as vesicles or high fever. In the group challenged with 200 ID₅₀ of FMDV, the body temperature of all of the PBS- or Ad5-LacZ-treated animals had increased to more than 40°C by 24 h postchallenge, whereas none of the animals exposed to Ad5-POL showed abnormalities at that time. In that group, one of five animals was fully protected. Moreover, some of the guinea pigs treated with FMDV-specific rAd5 had milder disease than the control animals, developing fewer and smaller vesicles, and recovering shortly thereafter (Table 1). These results indicate that adenovirus-delivered shRNAs have in vivo inhibitory activity against FMDV in guinea pigs.

TABLE 1. A single treatment with FMDV-specific rAd5 inhibits FMDV HKN/2002 infection in guinea pigs^a

Group	Treatment (PFU)	Challenge (ID ₅₀ /dpt)	No. protected/no. challenged for dpc							<i>P</i> ^b
			1	2	3	4	5	6	14	
PBS		50/1	3/5	0/5	–	–	–	–	–	AC
Ad5-LacZ	10 ⁷	50/1	5/5	1/5	1/5	0/5	–	–	–	0.0591
Ad5-NT21	10 ⁷	50/1	4/5	4/5	2/5	1/5	–	2/5 ^c	–	0.0326
Ad5-POL	10 ⁷	50/1	5/5	5/5	3/5	2/5	3/5 ^c	–	–	0.00149
Ad5-LacZ	10 ⁶	50/1	3/5	2/5	0/5	–	–	–	–	0.333
Ad5-NT21	10 ⁶	50/1	4/5	4/5	1/5	–	–	–	–	0.0326
Ad5-POL	10 ⁶	50/1	4/5	4/5	3/5	–	–	–	–	0.0326
Ad5-LacZ	10 ⁷	50/3	5/5	0/5	–	–	–	–	–	0.134
Ad5-NT21	10 ⁷	50/3	5/5	2/5	–	–	–	–	–	0.0571
Ad5-POL	10 ⁷	50/3	5/5	2/5	–	3/5 ^c	–	–	–	0.0316
PBS		200/1	3/5	0/5	–	–	–	–	–	AC
Ad5-LacZ	10 ⁷	200/1	4/5	2/5	0/5	–	–	–	–	0.173
Ad5-POL	10 ⁷	200/1	5/5	4/5	1/5	–	–	–	–	0.0116

^a –, Score is the same as the day before. dpt, days posttreatment.^b Statistical analysis by the log-rank test. AC, As controls.^c One animal recovered from the infection.

Kahana et al. (20) have shown that 100% inhibition of virus growth can be achieved in cells transfected with a mixture of three anti-FMDV siRNAs. This result prompted us to explore the potential of a single, or two successive, treatments with an FMDV-specific rAd5 mixture in guinea pigs. As shown in Table 2, no significant increase in antiviral effect was achieved in any of the groups with this form of treatment, compared to the groups treated with Ad5-NT21 or Ad5-POL alone (Table 1). This indicates that a simple combination of treatments cannot improve the protection of animals. Surprisingly, a control group inoculated once with the Ad5-NT21–Ad5-POL mixture and challenged immediately after inoculation had reduced susceptibility to virus infection: two of the five animals in this group were completely protected as determined by the absence of vesicles on their feet. This result suggests that rAd5 expressing FMDV-specific shRNA can rapidly prevent viral disease in animals.

Treatment with FMDV-specific shRNA-expressing rAd5 confers significant resistance against FMDV infection in swine. Based on the preliminary experiment in guinea pigs, we designed this part of the study to measure the antiviral activity of FMDV-specific rAd5 in swine, one of the major domestic animals that require serious consideration. Animals were intramuscularly inoculated with 4×10^9 PFU of rAd5 and challenged 24 h later with 100 ID₅₀ of HKN/2002. All of the

PBS-treated swine developed signs of FMD including vesicles by 4 days postchallenge (dpc) and fever for 2 to 3 dpc (Fig. 5A). Animal 99-12 had vesicles as early as 3 dpc, and the symptoms were much more severe 24 h later. In comparison with this group, animals inoculated with Ad5-LacZ showed no signs of significant antiviral activity, except that animal 104-2 developed vesicles 5 dpc, a little later than other animals (Fig. 5B). However, the Ad5-NT21–Ad5-POL mixture conferred marked antiviral activity that could be seen for all 21 days of observation (Fig. 5C). Animal 91-8 and 102-3 in this group developed no signs of FMD and were completely protected against viral challenge. Although 106-9 developed vesicles, these were fewer and developed much later. In another group inoculated with 8×10^9 PFU of Ad5-NT21–Ad5-POL mixture, only animal 107-2 was protected (Fig. 5D). Animals 93-10 and 104-3 showed delayed disease and milder disease than the control animals.

Serum samples were collected at 6, 14, and 21 dpc and tested for the presence of viremia, neutralizing antibodies, and antibodies against viral nonstructural protein 3ABC of FMDV. No animals had marked FMDV-specific antibodies at 0 dpc (data not shown), including anti-3ABC antibodies (Table 3). Animals in the control groups treated with either PBS or Ad5-LacZ developed viremia by 6 dpc and a high neutralizing antibody response for 14 to 21 dpc, suggesting that these ani-

TABLE 2. A single treatment or two treatments with an FMDV-specific rAd5 mixture does not increase protection of guinea pigs after FMDV infection^a

Group	Treatment	Challenge (dpt)	No. protected/no. challenged for dpc							<i>P</i> ^b
			1	2	3	4	5	6	14	
PBS	Single	1	4/5	0/5	–	–	–	–	–	AC
Ad5-LacZ	Single	1	5/5	1/5	1/5	0/5	–	–	–	0.180
Ad5-NT21–Ad5-POL	Single	1	5/5	4/5	3/5	2/5	3/5 ^c	–	–	0.00778
Ad5-NT21–Ad5-POL	Single	0	5/5	3/5	3/5	2/5	–	–	–	0.0411
Ad5-LacZ	Twice	0 ^d	5/5	2/5	0/5	–	–	–	–	0.0926
Ad5-NT21–Ad5-POL	Twice	0 ^d	5/5	3/5	2/5	2/5	–	–	–	0.0411

^a –, Score is the same as the day before. dpt, days posttreatment.^b Statistical analysis by the log-rank test. AC, as controls.^c One animal recovered from the infection.^d Animals were immediately challenged after the second inoculation.

FIG. 5. Antiviral activity of shRNA-expressing rAd5 in swine. Animals were treated with PBS (A), Ad5-LacZ (B), Ad5-NT21-Ad5-POL mixture (C), or a high dose of Ad5-NT21-Ad5-POL mixture (D) and challenged as described in Materials and Methods. After challenge, lesion scores were assigned to the animals according to the number of digits plus mouth with vesicles.

Animals were exposed to a very large amount of FMDV (Table 3). A very low neutralizing antibody response was detected in animals 91-8, 102-3, and 107-2 inoculated with the Ad5-NT21-Ad5-POL mixture, and all animals treated with 4×10^9 PFU of the Ad5-NT21-Ad5-POL mixture had no viremia at 6 dpc, suggesting that these animals effectively suppressed virus replication. Generally, viremia appears before clinical disease and lasts for 3 to 4 days (9, 28). Our results showed that most of the control animals developed disease at 4 to 5 dpc (Fig. 5), and all of the control animals had viremia at 6 dpc (Table 3). Thus, although we did not assay for that daily after challenge, the absence of viremia in animals 91-8, 102-3, and 106-9 at 6 dpc suggests the possibility that no viremia was present in the crucial period of challenge, or these animals just developed milder viremia and recovered quickly from that. Surprisingly,

animal 106-9 had viremia at 14 dpc as well as clinical disease 15 dpc. To our knowledge, swine will not develop FMD if they do not before 12 dpc because the challenge virus usually had already been cleared. It is therefore most likely that aerosol transmission of virus excreted by those sick animals should be responsible for this case, although they were housed in different rooms. A key distinguishing feature of infected animals is induction of antibodies against the polyprotein 3ABC of FMDV (34). Using an LPB-ELISA, we showed that most of the animals developing FMD in either control groups or experimental groups had significantly higher level of antibodies against 3ABC compared to the disease-free animals (Table 3).

DISCUSSION

In this study, we showed that rAd5 expressing both 1D-specific (Ad5-NT21) and 3D-specific (Ad5-POL) shRNA completely protected swine kidney IBRS-2 cells from homologous FMDV HKN/2002 infection; that Ad5-POL but not Ad5-NT21 prevented heterologous FMDV CHA/99 production in IBRS-2 cells; that when given by intramuscular injection to guinea pigs, both Ad5-NT21 and Ad5-POL rendered animals significantly less susceptible to FMDV; and that intramuscular treatment with an Ad5-NT21-Ad5-POL mixture conferred obvious resistance against FMDV infection in swine.

The short duration of the RNAi effect and the absence of complete viral clearance have been identified as challenges facing the use of RNAi as an effective antiviral agent (4). In a previous study (7) we showed that treatment with shRNA-expressing plasmids directed against the 1D gene of FMDV did not completely block virus multiplication in BHK-21 cells and that inhibition only lasted for ~ 48 h. Identical results were obtained in IBRS-2 cells transfected with plasmids expressing shRNAs targeting the 2B gene (10) and in BHK-21 cells transfected with synthetic siRNAs or siRNAs produced *in vitro* by using a Cocktail Kit (24, 27). However, Kahana et al. (20) demonstrated 100% inhibition of virus growth in BHK-21 cells

TABLE 3. Serological analysis of sera from swine inoculated with rAd5

Group	Animal no.	Viremia ^a		Neutralizing antibody ^b		Mean % inhibition of antibody against 3ABC ^c $\pm$ SD		
		Day 6	Day 14	Day 14	Day 21	Day 0	Day 14	Day 21
PBS	94-12	10.0	0	<256	256	10.9 $\pm$ 3.2	41.6 $\pm$ 4.0	58.1 $\pm$ 1.5
	99-12	2.2	0	256	<512	13.6 $\pm$ 3.8	45.0 $\pm$ 5.5	91.0 $\pm$ 2.5
	107-1	3.2	0	<512	512	19.4 $\pm$ 0.85	91.1 $\pm$ 0.1	112.1 $\pm$ 1.0
Ad5-LacZ	86-7	21.5	0	<256	256	19.7 $\pm$ 4.3	78.5 $\pm$ 5.7	87.7 $\pm$ 0.5
	93-3	2.2	0	<256	128	11.3 $\pm$ 6.5	58.0 $\pm$ 1.5	61.6 $\pm$ 0.9
	104-2	5.9	0	128	256	15.1 $\pm$ 4.0	50.8 $\pm$ 3.5	58.6 $\pm$ 4.8
Ad5-NT21-Ad5-POL	91-8	0	0	8	<16	11.6 $\pm$ 0.1	14.2 $\pm$ 8.8	22.5 $\pm$ 0.1
	102-3	0	0	8	<16	9.9 $\pm$ 2.0	30.1 $\pm$ 10.5	21.7 $\pm$ 0.7
	106-9	0	5.9	<16	<128	8.8 $\pm$ 4.2	32.4 $\pm$ 6.7	23.6 $\pm$ 0.6
Ad5-NT21-Ad5-POL ^d	93-10	ND ^e	ND	64	<256	9.1 $\pm$ 8.3	31.1 $\pm$ 8.1	77.6 $\pm$ 3.5
	104-3	ND	ND	<512	256	15.4 $\pm$ 1.7	37.1 $\pm$ 3.0	38.6 $\pm$ 1.1
	107-2	ND	ND	16	<32	10.4 $\pm$ 10.6	28.8 $\pm$ 2.9	37.9 $\pm$ 2.1

^a Infectious FMDV titers (TCID₅₀/0.1 ml) in the serum.

^b Highest dilution that resulted in a 50% reduction in the number of HKN/2002 plaques.

^c Mean percent inhibition of the optical density as determined by ELISA.

^d Inoculation with a high dose of rAd5 mixture.

^e ND, not detected.

transfected with a mixture of several anti-FMDV siRNAs. At present, we still lack understanding of the mechanisms that determine the antiviral efficiency of RNAi in cultured mammalian cells. In addition to differences in the efficiency of gene silencing due to differences in the structures of siRNAs and of their targets, we suggest that other layers of complexity need to be addressed, including (i) the extent of conservation of the RNAi machinery and its activity in many different mammalian cell types, (ii) the transfection efficiency of chemically synthesized and vector-derived siRNA, and (iii) the capacity of the RNAi effect to spread from one cell to another. The availability of high virus titers, the ability to infect a broad spectrum of cell types, and its lack of dependence on active cell division makes adenovirus the vector of choice for siRNA delivery (33, 38). In the present study, we have shown that virus progeny could not be detected in IBRS-2 cells treated with rAd5 expressing FMDV-specific shRNA from 48 h postchallenge (Fig. 3A and B), suggesting that virus replication was completely prevented.

In contrast to the success of RNAi in mammalian cell culture, there have been few reports of the effective use of siRNA in animal models (29). Given the unknown complexity of animals and the current lack of understanding of the molecular basis of RNAi in vertebrates, it is likely that the main challenge for developing in vivo RNAi is the delivery of duplex RNA intact to the target tissue. Therefore, as a first approach, we used adenoviruses as shRNA vectors in the present study. We found that guinea pigs were not completely protected when administered with FMDV-specific shRNA, even when inoculated with a high titer of rAd5 or receiving two inoculations. We further demonstrated that treatment with rAd5 only delayed the onset of disease in some animals. An important question is whether the adenovirus actually spreads throughout the tissues infected by the FMDV. In this connection we collected five organs (oropharynx, lung, liver, muscle, and epidermis of foot) from one of the Ad5-POL-treated and protected animals and one of the Ad5-LacZ-treated and unprotected animals in a repeat experiment at 8 dpc. Total RNAs were extracted from these tissues and assayed for rAd5 GFP mRNA and FMDV 3D mRNA by Q-RT-PCR. The vast majority of rAd5 was found in the liver (data not shown). However, most of the FMDV was located in the epithelial cells of the feet that contained vesicles, together with a minority of the rAd5. In cloven-hoofed animals, the oropharynx has been identified as the major site of FMDV replication during acute and persistent infection (31). Although we did not evaluate this in the swine in our study, the different tissue distribution of rAd5 and FMDV is probably responsible for the fact that treatment with rAd5 confers only limited inhibition of FMDV infection in vivo.

For it to be an effective antiviral agent in animals, it is essential that the RNAi signal should spread systemically. In *Caenorhabditis elegans* (15), siRNA induces systemic RNAi via a currently unknown mechanism. A multispanning transmembrane protein, SID-1, has been shown to mediate siRNA uptake and to be needed for systemic RNAi (14). Interestingly, SID-1 homologues exist in humans and mice (36). Recently, Duxbury et al. (13) have demonstrated that overexpression of a mammalian SID-1 homologue enhances siRNA uptake and gene-silencing efficiency in PANC1 human pancreatic duct ad-

enocarcinoma cells. This suggests that systemic RNAi may occur in higher species, including mammals. However, there is no recent evidence that an RNAi effect in one infected tissue can trigger a systemic antiviral response, and this may partially account for the modest antiviral activity of rAd5 in vivo. This question requires further exploration.

Another crucial issue that needs to be addressed is the optimal vector for delivery of shRNA-expressing cassettes. Replication-defective human adenovirus type 5 lacking E1A and E1B has been used by several groups (33, 38), including ourselves. However, adenovirus VA1 noncoding RNA is recently identified to be able to inhibit the biogenesis of siRNA and microRNA (25). Moreover, our results indicate that the antiviral potential of RNAi is somewhat impaired in swine treated with a high dose of adenoviruses (Fig. 5D). These findings suggest it is likely that these adenovirus vectors will need to be further modified, probably by deleting the VA1 gene (25), or even other novel siRNA delivery systems would be necessitated.

Since the discovery of the machinery of RNAi and its potential as a tool for the study of gene function and as a therapeutic platform, the practicality of RNAi especially in vivo has been of great interest. Although many problems of siRNA design, production, and delivery remain, progress can be expected from further animal studies.

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