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Application of RNA interference for inhibiting the replication of feline immunodeficiency virus in chronically infected cell lines

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Abstract

RNA interference (RNAi) is a process in which double-stranded RNA induces the post-transcriptional sequence-specific degradation of homologous messenger RNA. The present study was carried out to apply the RNAi technology to inhibit the replication of feline immunodeficiency virus (FIV). Four small interfering RNAs (siRNAs) homologous to the FIV *gag* gene were synthesized and transfected into a feline fibroblastic cell line chronically infected with FIV (CRFK/FIV). These synthetic siRNAs efficiently inhibited the replication of FIV. Next, we examined the effect of retroviral vector-mediated transfer of FIV-specific short hairpin RNA (shRNA) on the replication of FIV in a feline T-cell line chronically infected with FIV (FL4). The retroviral vector-mediated transfer of FIV-specific shRNA was shown to markedly inhibit the replication of FIV in the FL4 cells. These results provide useful information for the development of RNAi-based gene therapy strategy to control FIV infection.
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Keywords: Feline immunodeficiency virus; Small interfering RNA; Short hairpin RNA; Retrovirus vector

1. Introduction

Feline immunodeficiency virus (FIV) belongs to the genus *lentivirus* of the family retroviridae. FIV was first isolated in 1987 in the United States and has been reported to induce crucial immunodeficiency in infected cats, which is similar to that in human immunodeficiency virus (HIV)-infected people (Pedersen et al., 1987). FIV is now widely spread among domestic cats throughout the world including Europe, the United States, Australia, Vietnam, Taiwan and Japan (Ishida et al., 1989; Lin et al., 1995; Malik et al., 1997; Nakamura et al., 2000; Steinrigl and Klein, 2003; Yamamoto et al., 1989). FIV-infected cats show various degree of immunological impairment resulting in the clinical symptoms including gingivitis,

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stomatitis, upper respiratory tract infections, myelosuppression and opportunistic infections (Bendinelli et al., 1995; Pedersen and Barlough, 1991; Shelton et al., 1990). Because of the prevalence and various clinical outcomes, FIV infection has been considered as one of the most problematic diseases in small animal practice. A number of antiviral drugs such as reverse transcriptase inhibitors and protease inhibitors, have been shown to inhibit FIV replication in experimentally and naturally FIV-infected cats (Arai et al., 2002; Egberink et al., 1990; Hartmann et al., 1992). However, severe hematological side effects of their antiviral drugs and insufficient clinical improvements in FIV-infected cats were also reported (Arai et al., 2002; Hartmann et al., 1992). Therefore, no effective antiviral therapy for FIV-infected cats has been established.

RNA interference (RNAi) is a process of sequence-specific, post-transcriptional gene silencing (PTGS) triggered by double-stranded RNA (dsRNA) homologous to the target transcripts (Fire et al., 1998). The dsRNAs are processed by RNase III-like enzyme Dicer into small interfering RNAs (siRNAs) of 21–23 nucleotides (Bernstein et al., 2001). These siRNAs form an RNA-induced silencing complex (RISC), which leads to the degradation of homologous target RNA (Zamore et al., 2000). Introduction of siRNAs can induce the sequence-specific gene silencing in mammalian cells (Elbashir et al., 2001). Recent studies have demonstrated that RNAi can be exploited to target a variety of human viruses including HIV-1, poliovirus, human papillomavirus, hepatitis C virus (HCV), hepatitis B virus (HBV), influenza virus and severe acute respiratory syndrome (SARS)-coronavirus (Ge et al., 2003; Gitlin et al., 2002; Jacque et al., 2002; Jiang and Milner, 2002; Randall et al., 2003; Shlomai and Shaul, 2003; Wang et al., 2004). These findings indicate that RNAi represents a promising gene therapeutic approach to control viral infection.

Most important problems of siRNA are the low efficiency of transfection in primary cells and its transient inhibitory effect (Elbashir et al., 2001). In contrast, endogenous expression of short hairpin RNA (shRNA) mediated by viral vectors has been shown to induce persistently the sequence-specific gene silence in a variety of primary cells as well as in experimental animals (Rubinson et al., 2003; Xia et al., 2002). To use the RNAi technology as a strategy of gene therapy for the control of FIV infection, both development of effective delivery method of siRNAs into feline lymphoid cells and achievement of sustained inhibitory effect are required. Lentivirus vector is now recognized to be the most efficient delivery method into non-dividing primary cells. However, it has not been used for clinical practices because the safety of lentivirus vector-mediated gene transfer has not been fully established. On the other hand, retrovirus vector has been applied for several human gene therapy clinical trials (Edelstein et al., 2004). There have been a number of reports indicating the therapeutic effect of retrovirus vector-mediated transfer of antiviral genes in HIV-infected people (Deeks et al., 2002; Wong-Staal et al., 1998).

In this study, to investigate the potential of RNAi as a therapeutic strategy for the control of FIV infection, we first examined the effect of chemically synthesized siRNAs homologous to FIV *gag* gene on the replication of FIV in chronically FIV-infected fibroblastic cells. Next, to deliver FIV-specific shRNA into feline lymphoid cells efficiently and to achieve persistent inhibitory effect on the replication of FIV in these cells, we generated a FIV-specific shRNA expression retrovirus vector and examined its effect on the replication of FIV in a chronically FIV-infected T-cell line.

2. Materials and methods

2.1. Cells

A feline fibroblastic cell line (CRFK) chronically infected with FIV Petaluma strain (CRFK/FIV) was maintained in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (FCS) and penicillin/streptomycin. A feline T-cell line, FL4, chronically infected with FIV Petaluma strain was maintained in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated FCS and penicillin/streptomycin.

2.2. Preparation of siRNAs

The design of siRNAs was based on general guidelines (Elbashir et al., 2001). Four siRNAs (G1, G2, G3, and G4) corresponding to *gag* gene of

Petaluma FIV14 (Olmsted et al., 1989) (GenBank accession number: M25381) were chosen from the highly conserved sequence of FIV *gag* gene (Kakinuma et al., 1995) and synthesized (Dharmacon Research, Lafayette, CO). An siRNA (Luc) corresponding to the coding sequence of firefly luciferase was purchased from Dharmacon Research and used as a control. Sequences of the sense and antisense strands of siRNAs targeted to FIV *gag* gene are shown in Table 1. The siRNA strands were deprotected and annealed according to the manufacture's instructions.

2.3. Transfection of synthetic siRNAs

CRFK/FIV cells were trypsinized and plated at 2×10^5 cells per well (9.6 cm^2) in six-well plates 24 h prior to siRNA transfection in DMEM containing 10% FCS without antibiotics. Cationic lipid complexes, prepared by incubating the appropriate amount of indicated siRNA (0.04, 0.2 or 1.0 μM) with 3 μl of Oligofectamine (Invitrogen, Rockville, MD) in 100 μl of Opti-MEM-I medium (Invitrogen) for 20 min, were added to the wells in a final volume of 1 ml. After incubation for 4 h, the cells were washed, and then cultured in DMEM containing 10% FCS for further experiments.

2.4. Vector construction

A murine leukemia virus (MLV)-based vector (pSIREN-retroQ: BD Clontech, Palo Alto, CA) containing a human U6 RNA polymerase III promoter driving the expression of shRNA and a puromycin resistance gene expression cassette was used in this study. shRNA oligonucleotides were designed to contain a sense strand of 19-nucleotide sequences followed by a short spacer (TTCAAGAGA), a reverse complement of the sense strand and six thymidines as an RNA polymerase III transcriptional stop signal. To generate FIV *gag* gene-specific shRNA, two complementary DNA oligonucleotides were annealed and inserted between *Bam*HI and *Eco*RI sites immediately downstream of U6 promoter (pU6shG4). The pU6shG4 contained siRNA sequence corresponding to *gag* gene of FIV Petaluma strain (nt 1626–1644 in FIV14 genome). Sequences of the sense and antisense strands are 5′-GATCCGCCAGAAAGTACCCTA-GAATTCAAGAGATTCTAGGGTACTTTCTGGCT TTTTTG-3′ and 5′-AATTCAAAAAAGCCAGAAA GTACCCTAGAATCTCTTGAATTCTAGGGTACTT TCTGGCG-3′, respectively. A negative control vector, pU6shNC, containing siRNA sequence non-homologous to mammalian genes was also generated by using a sense oligonucleotide, 5′-GATCCGT GCGTTGCTAGTACCAACTTCAAGAGAGTTGG-TACTAGCAACGCACTTTTTTG-3′ and its antisense oligonucleotide, 5′-AATTCAAAAAAGTGCGTTGC TAGTACCAACTCTCTTGAAGTTGGTACTAGCA ACGCACG-3′ (RNAi-Ready pSIREN-RetroQ Vector Kit: BD Clontech). All of the constructs were verified by nucleotide sequencing. Vesicular stomatitis virus envelope expression plasmid, pVSV-G (BD Clontech), was used to generate the vector virus that could infect feline cells.

Table 1
Sequences of siRNAs and corresponding regions in genome of FIV14 clone

siRNA	Sequence	Location in FIV14 genome (nt)
G1		
Sense	5′-GAGGAAGGCCCUCCACAGGdTdT-3′	1009–1027
Antisense	5′-CCUGUGGAGGGCCUUCCUCdTdT-3′	
G2		
Sense	5′-CACCUACUGACAUGGCCACdTdT-3′	1172–1190
Antisense	5′-GUGGCCAUGUCAGUAGGUGdTdT-3′	
G3		
Sense	5′-GCAAGAUUUGCACCAGCUAdTdT-3′	1366–1384
Antisense	5′-UAGCUGGUGCAAAUCUUGCdTdT-3′	
G4		
Sense	5′-GCCAGAAAGUACCCUAGAAdTdT-3′	1626–1644
Antisense	5′-UUCUAGGGUACUUUCUGGCdTdT-3′	

2.5. Retrovirus vector production

GP2-293 cell line (BD Clontech) is a human embryonic kidney (HEK293)-derived packaging cell line that stably expresses *gag* and *pol* gene of MLV. The cell line was cultured in DMEM supplemented with 10% heat-inactivated FCS, penicillin/streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate. To generate VSV-G pseudotyped MLV-based shRNA expression retrovirus vectors, GP2-293 cells in 75 cm^2 flask were cotransfected with a mixture of 15 μg of pVSV-G and 15 μg of pU6shG4 or pu6shNC by using cationic lipid complex method (Lipofectamine 2000: Invitrogen). After incubation at 37 °C for 4 h, the medium was removed, and the cells were washed with phosphate buffered saline (PBS), and then cultured in fresh medium. The culture supernatants containing the produced viruses were harvested 24, 48 and 72 h after transfection, filtrated through a 0.45 μm-pore size filter, and then centrifuged at 50,000 × *g* at 4 °C for 90 min. The viral pellets were resuspended in serum-free DMEM in 1/100 of the initial volume at 4 °C overnight. Aliquots of the concentrated viruses were stored at −80 °C for subsequent use. The concentrated virus stocks were titrated in NIH3T3 cells by colony forming assay. NIH3T3 cells (1×10^5 cells) in DMEM containing 10% heat-inactivated FCS were seeded into wells (9.6 cm^2) of six-well culture plates. After incubation at 37 °C for 12 h, the cells were infected with 1 ml of the serial dilutions of each virus stock in the presence of 8 μg of polybrene (Sigma) per ml. After incubation at 37 °C for 48 h, the medium containing viruses were removed, and the cells were washed twice with PBS, and then cultured for 10 days in the presence of 1 μg of puromycin (BD Clontech) per ml. The titers of the virus stocks were prepared to be (2–4) × 10^7 cfu/ml.

2.6. Retrovirus vector transduction

FL4 cells (2×10^5 cells/well) were seeded into wells (3.8 cm^2) of a 12-well plate coated with 10 μg of Retronectin (TaKaRa Shuzo, Kyoto, Japan) per cm^2 and inoculated with the retrovirus vectors at a multiplicity of infection (MOI) 10 in a final volume of 1 ml of RPMI 1640 medium containing 10% heat-inactivated FCS. After incubation at 37 °C for 48 h, the cells were washed twice with PBS, and then cultured in the presence of 1 μg of puromycin per ml for 10 days to generate its stably transduced cells.

2.7. Real-time sequence detection system

Total cellular RNAs were isolated from cells by using guanidine isothiocyanate method (RNeasy Mini Kit, QIAGEN, Valencia, CA) according to the manufacturer's protocol. The sequences of primers and probes used in this study are previously reported (Goto et al., 2002; Mizuno et al., 2003) and are as follows: a forward primer 413F (5′-AAACAG-TAAATGGAGCACCACAGTAT-3′, nucleotides (nt) 1040–1065 in pFIV14), a reverse primer 495R (5′-TAGCCCCTCTCTTGCCTTCTC-3′, nt 1122–1102), an internal probe 440T (5′-TAGCACTTGACC-CAAAAATGGTGTCCAATT-3′, nt 1067–1095), a forward primer FBA2S (5′-TGCTGTCTCTGTA CGCTTCTGG-3′, nt 228–249 in feline *β-actin* mRNA), a reverse primer FBA2R (5′-TGTG GGTGACCCCGTCC-3′, nt 287–270) and a internal probe FBATP (5′-CGCACCACTGGTATTGTCATG-GACTCTG-3′, nt 251–268). The probes were labeled with a reporter dye, 6-carboxyfluorescein (FAM) and a quencher dye, 6-carboxytetramethylrodamine (TAMRA) at their 5′ and 3′ ends, respectively. Total RNA from each sample was subjected to reverse transcription and polymerase chain reaction (PCR) amplification with a TaqMan Gold RT-PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. In brief, a master mixture containing all reagents required for RT-PCR was prepared: 1× TaqMan buffer A, 0.2 μM dNTPs, 5 μM magnesium chloride, 0.25 U of MultiScribe reverse transcriptase per μl, 0.4 U of RNase inhibitor per μl, and 0.05 U of AmpliTaq Gold DNA polymerase per μl. Total RNA extract (200 ng of total RNA per tube) was added to the master mixture. This mixture was used for assays of FIV RNA and *β-actin* mRNA used as an internal control to give a final probe concentration of 200 nM and a final concentration of each primer of 900 nM, and the total reaction volume was adjusted to 50 μl. The target and internal control were reverse transcribed at 48 °C for 30 min, held at 95 °C for 10 min to activate AmpliTaq Gold, and subjected to PCR consisting of 40 cycles of denaturation at 95 °C for 15 s and annealing/polymerization at 60 °C for 1 min, with a ABI PRISM7700

sequence detector (Applied Biosystems). The PCR cycle number at the threshold line was represented as C_T. The difference between C_T for the target and C_T for the internal control, ΔC_T, was calculated. The ΔC_T values were used to estimate the amounts of FIV RNA. According to the manufacturer's instructions, $2^{-\Delta C_T}$ values were calculated, giving a predicted amount of FIV RNA in comparison to that of *β-actin* mRNA.

2.8. Reverse transcriptase (RT) activity assay

Ten microliters of the cell-free culture supernatant was added to 50 μl of RT buffer containing 5 μg of poly (A) (Sigma) per ml, 0.16 μg of oligo (dT) (Roche Diagnostics, Indianapolis, IN) per ml, 0.1% Nonidet NP-40, 60 mM Tris–HCl, 75 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 4 mM DTT and 10 μCi of [^{32}P] dTTP (Amersham Biosciences, Piscataway, NJ) per ml. After incubation at 37 °C for 3 h, 10 μl of the mixtures were spotted onto DEAE filter papers (Wallac Oy, Turku, Finland) and washed four times with 2× SSC (1× SSC is 0.15 M sodium chloride plus 15 mM sodium citrate) and once with 99% ethanol. The filter papers were then dried, and the incorporated radioactivity on the filter paper was measured with autoradiography. RT-activity per ml of culture supernatant was calculated from the value of incorporated radioactivity per 10 μl of each reaction mixture.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) was used to test for a significant difference in each RT activity in the culture supernatants. If a significant difference was found by ANOVA, Tukey–Kramer's honestly significant difference test was performed to determine which pair showed the difference. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Effect of synthetic siRNAs on the replication of FIV in CRFK/FIV cells

We examined the effect of four siRNAs homologous to *gag* gene of FIV Petaluma FIV14 clone (G1, G2, G3, and G4) on the replication of FIV in CRFK/FIV cells.

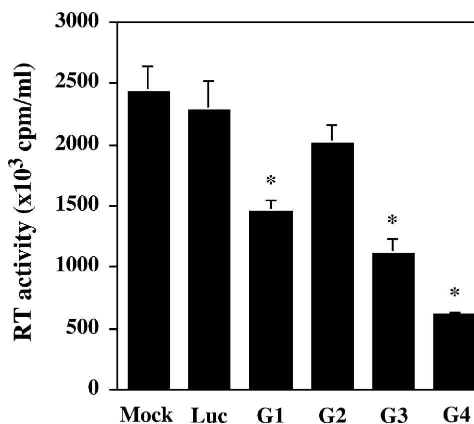

Fig. 1. Effect of siRNAs on the replication of FIV in CRFK/FIV cells. The RT activity in the culture supernatants was measured 48 h after transfection with each siRNA at a concentration of 200 nM. The columns and bars show means and standard deviations, respectively, obtained from the data in triplicate samples. Asterisks indicate statistically significant difference between transfection with anti-FIV siRNAs and Luc-siRNA as a control ($P < 0.05$).

An siRNA homologous to firefly *luciferase* (Luc) was used as a control. RT activity in the culture supernatant 48 h after transfection was significantly ($P < 0.05$) reduced in the cells transfected with G1, G3 or G4-siRNA compared to those in mock-transfected and anti-Luc siRNA-transfected cells (Fig. 1). Among the four anti-FIV siRNAs, G4-siRNA showed the strongest inhibitory effect on the replication of FIV.

3.2. Dose-dependent effect of FIV-specific siRNA on the replication of FIV in CRFK/FIV cells

To evaluate the dose-dependency of the inhibitory effect of anti-FIV siRNA on the replication of FIV, we examined the effect of G4-siRNA on the replication of FIV in CRFK/FIV cells at various concentrations. Transfection with G4-siRNA resulted in significant ($P < 0.05$) reduction of the amount of FIV RNA in the cells in a dose-dependent manner whereas those in the cells transfected with Luc-siRNA at any concentrations were not different from that in mock-transfected cells (Fig. 2a). Corresponding to the reduction of the amount of FIV RNA, RT activity in the culture supernatant 48 h after transfection with G4-siRNA was also reduced in a dose-dependent manner (Fig. 2b). These results indicated that siRNA homologous to FIV *gag* gene could specifically

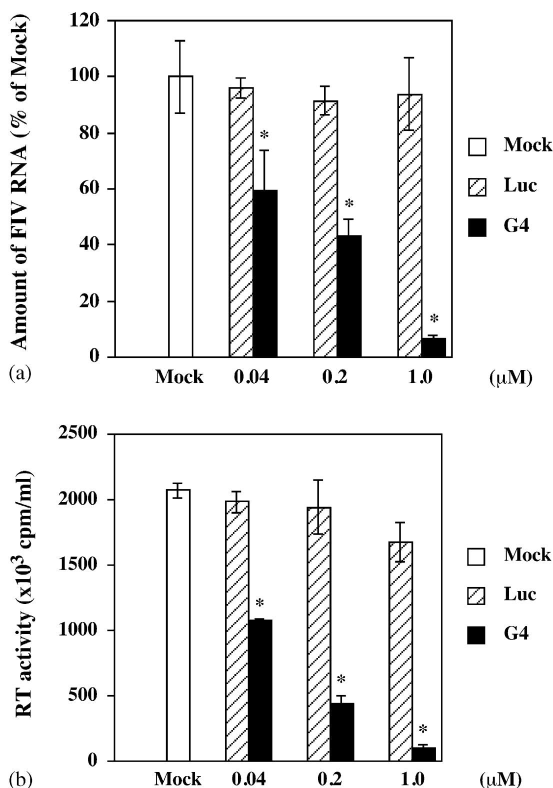

Fig. 2. Dose-dependency of the effect of FIV-specific siRNA on the amount of FIV RNA in CRFK/FIV cells. The amount of FIV RNA in the cells (a) and RT activity in the culture supernatants (b) were measured 48 h after transfection with G4-siRNA or Luc-siRNA (control) at concentrations as indicated. The amount of FIV RNA was measured by a real-time sequence detection system and the relative FIV RNA expression values ($2^{-\Delta C_T}$) were calculated in comparison to the amount of *β-actin* mRNA. The amounts of FIV RNA are shown as percentages of the amount of FIV RNA in mock-transfected cells. The columns and bars show means and standard deviations, respectively, obtained from the data in triplicate samples. Asterisks indicate statistically significant difference between transfection with G4-siRNA and that with Luc-siRNA at same concentration ($P < 0.05$).

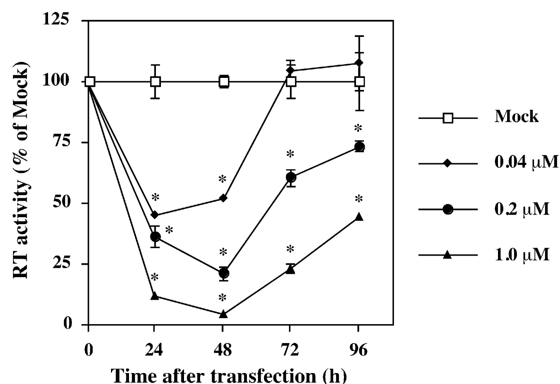

Fig. 3. Time-course analysis of the inhibitory effect of FIV-specific siRNA on the replication of FIV in CRFK/FIV cells. RT activity in the culture supernatants was measured at several time points as indicated after transfection with G4-siRNA at concentrations of 0.04 μM (◆), 0.2 μM (●) and 1 μM (▲). RT activity in the culture supernatants are shown as percentages of RT activity in the mock-transfected cells (□). The symbols and bars show means and standard deviations, respectively, obtained from the data in triplicate samples. Asterisks indicate statistically significant difference between transfection with G4-siRNA and mock-treated cells ($P < 0.05$).

inhibit the replication of FIV in chronically FIV-infected cells.

3.3. Time-course analysis of the inhibitory effect of FIV-specific siRNA on the replication of FIV in CRFK/FIV cells

Next, to evaluate the time-course of the inhibitory effect of anti-FIV siRNA on the replication of FIV, the replication of FIV in CRFK/FIV cells transfected with G4-siRNA was examined at several time points. RT activity in the culture supernatant was lowest 48 h after transfection (78% and 96% reduction at 200 nM and 1 μM, respectively) and then gradually increased (Fig. 3). These results indicated that the inhibitory effect of siRNA homologous to FIV *gag* gene was transient in chronically FIV-infected cells.

3.4. Effect of retrovirus vector-mediated transfer of FIV-specific shRNA on the replication of FIV in FL4 cells

The effect of retrovirus vector-mediated transfer of FIV-specific shRNA on the replication of FIV was investigated in FIV-infected T cells. FL4 cells, feline T-cell line chronically infected with FIV Petaluma strain, were inoculated with VSV-G pseudotyped retrovirus vector with a construct to express FIV-specific shRNA, and stably shRNA-expressing cells were selected by cultivation in the presence of puromycin. Replication of FIV in these cells was examined 2 weeks after the inoculation with the retrovirus vectors.

The amount of FIV RNA was significantly ($P < 0.05$) smaller in the cells infected with the

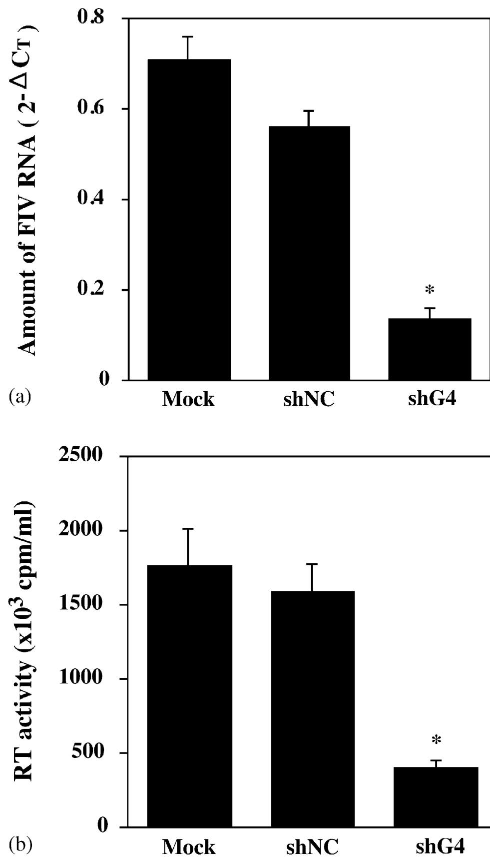

Fig. 4. Effect of retrovirus vector-mediated transfer of FIV-specific shRNA on the replication of FIV in FL4 cells. FL4 cells were infected with the retrovirus vector, which express shG4 or shNC and then selected under puromycin. The amount of FIV RNA in the untreated, shG4-transduced, and shNC-transduced FL4 cells (a) and RT activities in the culture supernatants (b) were measured 2 weeks after retrovirus vector-mediated transduction. The amount of FIV RNA was measured by a real-time sequence detection system and the relative FIV RNA expression values ($2^{-\Delta C_T}$) were calculated. The columns and bars show means and standard deviations, respectively, obtained from the data in triplicate samples. The asterisk indicates statistically significant difference between cells transduced with shG4 and shNC ($P < 0.05$).

retrovirus vector with a construct to express shRNA specific to FIV (shG4) ($2^{-\Delta C_T}$ value, 0.138 ± 0.024) than in untreated cells (0.709 ± 0.051) and the cells infected with the control retrovirus vector expressing shRNA non-homologous to mammalian genes (shNC) (0.559 ± 0.035) (Fig. 4a).

Furthermore, RT activity in the culture supernatant was also significantly ($P < 0.05$) reduced in the cells infected with the retrovirus vector expressing shG4 (404 ± 45.9 cpm/μl) compared to that in untreated cells (1758 ± 251 cpm/μl) and the cells infected with the retrovirus vector expressing shNC (1592 ± 177 cpm/μl) (Fig. 4b).

4. Discussion

RNAi technology has been considered as a new therapeutic strategy for a variety of viral infections in humans. It has been demonstrated that inhibition of the replication of HIV-1 and other viruses can be achieved by introduction of siRNA or shRNA in both cultured cell lines and primary cells. The present study was carried out to demonstrate a potential of RNAi technology as a gene therapy strategy for the control of FIV infection.

To identify the sequence of siRNA, which can efficiently inhibit the replication of FIV, we examined the effect of four siRNAs homologous to FIV *gag* gene on FIV replication in CRFK/FIV cells. We demonstrated that transfection with anti-FIV siRNAs (G1, G3 and G4) resulted in reduction of RT activity in the culture supernatant, indicating that a diverse region of FIV *gag* gene can be targeted by siRNA. For application of RNAi technology to therapeutic purposes of naturally FIV-infected cats, sequence diversity of FIV genome should be considered. The sequence used for preparation of G4-siRNA, which efficiently inhibited the replication of FIV in this study, is highly conserved among various strains of FIV (Kakinuma et al., 1995). Therefore, it is highly plausible that the siRNA such as G4 inhibits the replication of various field strains of FIV as well as Petaluma strain. However, since gene silencing by RNAi requires almost perfect complementarity between the siRNA and its target sequence, further investigation is required to examine the inhibitory effect of the siRNAs on the replications of diverse FIV strains.

In this study, we showed that G4-siRNA inhibited the replication of FIV in a dose-dependent manner, whereas Luc-siRNA did not. These results indicated that siRNA homologous to FIV *gag* gene could specifically inhibit the replication of FIV in chronically FIV-infected cells. Transfection with G4-siRNA at a higher concentration (1 μM) resulted in inhibition of the replication of FIV by 96%, whereas its inhibition was by 48% at a lower concentration

(40 nM). As a large amount of viral transcripts would be generated *de novo* in CRFK/FIV cells, enough amount of siRNAs might be required to efficiently inhibit the replication of FIV in the cells.

When siRNAs were transiently transfected into CRFK/FIV cells, the strongest inhibitory effect on the viral replication was observed at 48 h after transfection, then its effect was gradually decreased. This result is consistent with previous reports describing that the silencing effect of siRNA rapidly faded away in continuously dividing mammalian cell lines (Elbashir et al., 2001). On the other hand, Song et al. demonstrated that inhibition of the replication of HIV-1 in non-dividing primary monocyte-derived macrophages by transfection with siRNA targeted HIV p24 sustained at least 15 days after transfection (Song et al., 2003). As CRFK/FIV cells were rapidly proliferating, the short-term effect of siRNAs shown in this study might be due to the dilution of siRNAs in the cells.

The low transduction efficiency of siRNAs into T cells and its transient effect are the most important problems to use the RNAi technology as a strategy of gene therapy for the control of FIV infection. Retrovirus vector can infect primary cells and induce the sustained expression of transferred gene. Therefore, we examined the effect of the retrovirus vector-mediated transfer of the shRNA in feline T-cell line. The amount of FIV RNA and RT activity in the culture supernatant of the FL4 cells were markedly decreased even 2 weeks after transduction with a retrovirus vector which express shRNA specific to FIV. This result suggests the therapeutic effect of the retrovirus vector-mediated delivery of FIV-specific shRNA in cells infected with FIV. However, the retrovirus vector-mediated FIV-specific shRNA transfer could not achieve complete inhibition of FIV replication even after the selection by antibiotic. As for the reason, there might exist a small number of cells in which the amount of shRNA was not enough to inhibit FIV replication even in cells after antibiotic selection. As another possibility, FIV clones to escape from the shRNA might exist in FL4 cells because it is not a cloned cell line.

The goal of the gene therapy strategy for the control of FIV infection is to obtain FIV-resistant T-lymphocytes. However, the transduction efficiency of a same retrovirus vector used in this study containing a fluorescence marker gene, *ZsGreen*, into feline peripheral blood mononuclear cells was 5–6% at MOI 10 whereas more than 90% into FL4 cells (data not shown). Further investigation is necessary to improve the transduction efficiency of the retrovirus vector in primary T cells. Another candidate of target cells for RNAi gene therapy to control FIV infection is hematopoietic stem cells (HSC). Development of antiviral gene therapy strategies that target HSC has been proposed as a long-term treatment for AIDS induced by HIV infection. In theory, permanent resistance to HIV infection can be achieved through the introduction of antiviral genes into pluripotent HSC because of their capacity of self-renewal as well as proliferation and differentiation into lymphoid lineage. Recently, HSCs transduced with siRNA construct directed to HIV were shown to allow to mature into T-lymphocytes *in vivo* in SCID-hu mouse thy/liv grafts and these HSC-derived siRNA-expressing T cells and macrophages showed marked resistance to HIV infection (Banerjea et al., 2003). Although isolation and purification system of feline HSC has not been established, viral vector-mediated transfer of anti-FIV shRNA into HSC will be a useful gene therapy strategy for the control of FIV infection in cats in future.

In summary, we demonstrated that siRNAs homologous to FIV *gag* gene efficiently inhibited the replication of FIV in fibroblastic cells chronically infected with FIV. Moreover, we showed that retrovirus vector-mediated transfer of FIV-specific shRNA could inhibit the replication of FIV in lymphoid cells chronically infected with FIV. The present study indicates that RNAi technology can be useful as a gene therapy strategy for the control of FIV infection.

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