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Inhibition of West Nile Virus Replication by Retrovirus-Delivered Small Interfering RNA in Human Neuroblastoma Cells

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Abstract

West Nile virus (WNV) has been responsible for the largest outbreaks of arboviral encephalitis in U.S. history. No specific drug is currently available for the effective treatment of WNV infection. To exploit RNA interference as a potential therapeutic approach, a Moloney murine leukemia virus-based retrovirus vector was used to effectively deliver WNV-specific small interfering RNA (siRNA) into human neuroblastoma HTB-11 cells. Viral plaque assays demonstrated that transduced cells were significantly refractory to WNV replication, as compared to untransduced control cells ($P < 0.05$), which correlated with the reduced expression of target viral genes and respective viral proteins. Therefore, retrovirus-mediated delivery of siRNA for gene silencing can be used to study the specific functions of viral genes associated with replication and may have potential therapeutic applications.

Keywords

WNV; small interfering RNA (siRNA); retroviral vector; gene therapy

INTRODUCTION

Like other mosquito-borne human pathogenic viruses (such as yellow fever virus and dengue viruses), West Nile virus (WNV) is an enveloped RNA virus belonging to the genus *Flavivirus* in the family *Flaviviridae*. WNV contains a single plus-sense RNA genome of approximately 11 kb, which encodes a single long open reading frame. The encoded polypeptides are co- and post-translationally processed by viral and cellular proteases into three structural proteins [capsid (C), premembrane (prM), or membrane (M), and envelope (E) protein] and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) [Rice, 1996; Sampson et al., 2000; Brinton, 2002]. WNV has only recently been recognized as a major human pathogen, following outbreaks of human encephalitis reported in Romania, Russia, Israel, and in particular, New York City in 1999 [Anderson et al., 1999; Lanciotti and

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Roehrig, 1999; Savage et al., 1999; Lvov et al., 2000; Hindiyeh et al., 2001; Mazurek et al., 2005]. Subsequently, WNV has spread throughout the continental United States, sparing only Alaska and Hawai'i. The U.S. epidemic in 2003 was the largest WNV outbreak ever reported, with 9,862 cases of infection, including 2,860 (29%) cases of meningitis or encephalitis, reported to the Centers for Disease Control and Prevention (CDC). In 2006, 9,794 cases of neuroinvasive WNV infection (including 934 fatalities) were reported. Currently, no effective drugs or vaccines are available to treat or prevent WNV infection in humans.

The phenomenon of RNA interference (RNAi) is a cellular process that degrades specifically RNA within the cytoplasm of cells in a sequence-specific manner [Elbashir et al., 2001; Agami, 2002; Paddison et al., 2002]. RNAi occurs naturally in plants, nematodes, parasites, insects, and mammalian cells, and is believed to function as a regulator of cellular gene expression and a possible innate defense against viral infections [Jorgensen, 1990; Romano and Macino, 1992; Fire et al., 1998; Agami, 2002; Gitlin et al., 2002; Paddison et al., 2002].

The use of small interfering RNA (siRNA) has become a powerful tool to down-regulate or knock down the expression of specific genes in mammalian cells, as well as in whole organisms [Elbashir et al., 2002; Paddison et al., 2002; Sui et al., 2002]. Furthermore, many mammalian viruses appear susceptible to treatment with exogenous siRNA [Gitlin et al., 2002; Lindenbach and Rice, 2002; Novina et al., 2002; Hall and Alexander, 2003; Kapadia et al., 2003; McCaffrey et al., 2003; Wilson et al., 2003; Wiebusch et al., 2004; Zhang et al., 2004; Ong et al., 2006; Weinberg et al., 2007]. This sequence-specific activity of siRNA against viruses has led to a great deal of interest in its potential use as a new approach toward antiviral therapy [Carmichael, 2002; Cullen, 2002; Lindenbach and Rice, 2002; Shuey et al., 2002; Silva et al., 2002].

Plasmid- and oligonucleotide-based systems possess the potential for widespread use. However, they are limited by their instability and inability for long-term use, as well as their dependence on transfection. Only certain cell lines can be transfected efficiently. In contrast, retroviral gene delivery is effective in most cell lines, including the many primary cell types and fertilized eggs used in generating transgenic animals [Barquinero et al., 2004]. Experimental data demonstrate that efficient inhibition of WNV replication can be achieved in the human neuroblastoma cell line HTB-11 through use of a Moloney murine leukemia virus (MMLV)-based retroviral delivery system of WNV-specific siRNA.

MATERIALS AND METHODS

siRNA Sequence Selection and Construction of Short Hairpin RNA Expressing Retroviral Vector: N2A-SV40-GFP-siRNA

The human H1-RNA *pol* III promoter (−328 to −1) was amplified from human genomic DNA with a pair of primers (5'-TGTGATAGATCTCCATGGAATTCGAACGCTGACGTC-- and 5'-TTCTACGCGTGCTTGGATCCGAGTGGTCTCATACAGAACTTATAAGATTCC-3') in which a *Bam* HI site was introduced at the 3' end, allowing for insertion of the siRNA sequences at the −1 position of the H1 transcript. The PCR fragment was cloned at the *Bgl* II and *Mlu* I sites of the MMLV retroviral vector plasmid N2A-SV40-GFP (9,421 bp), a newly modified N2A-based vector in this laboratory by replacing the *Neo* with eGFP and inserting a SV40 ori downstream of 3'LTR for enhanced viral vector replication in cells expressing SV40 large T antigen.

Five specific sequences of siRNA, based on the genome of the WNV (NY-99, flamingo), were selected using the online program siRNA Target Finder (Ambion, Austin, TX). The siRNA were targeted to different regions of the structural and nonstructural genes: 5-GAACAAACAAACAGCGATG-3 (nt 312–330), 5-GACGCAGTCGGAGGTC-3 (nt 728–746), 5-GGAGTGTCTGGAGCAACAT-3 (nt 1005–1023), 5-GCTAAGGTGCTTGA

GCTGC-3 (nt 9335–9353), 5-GAACCGTCATGGATGTTAT-3 (nt 9447–9465) (Fig. 1). One sequence that contained a mismatch (nucleotides are underlined) from the original target site, (nt 312–330) 5-GAAGAAAGAAAGACCGATG-3, was selected as a negative control. All of these sequences were analyzed using the BLAST search program of the GenBank database to avoid analogous sequences found in the human genome. Oligonucleotides with these sequences, along with complementary strands, were synthesized, annealed, and subcloned into the MMLV retroviral-vector backbone between the *Mlu* I and *Bam* HI sites, under the control of the human H1 promoter.

Cells and Viruses

Human embryonic kidney cell line 293-T was obtained from ATCC and maintained in DMEM with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and used for vector production. Human T-cells (CEM) were grown in RPMI1640 medium supplemented with 10% FBS and antibiotics and employed for vector titration. African green monkey kidney Vero cells which were employed for WNV titration and human neuroblastoma cell line HTB-11 were maintained in MEM with 10% FBS, 100 µg penicillin and 100 U streptomycin. WNV strain NY-99 flamingo was propagated and plaque titrated in Vero cells.

Generation of Vector Virus, Infection, and siRNA Expression

293-T cells at exponential growth phase in a TC-150 cm² flask were transfected by calcium phosphate precipitation with 5 µg pVSV-G (CLONTECH, Mountain View, CA), 12.5 µg N2A-SV40-GFP-siRNA and 12.5 µg of packaging plasmid pAmpho provided by Dr. V. Planelles from University of Utah at Salt Lake City. Cell medium was replaced 8 hr post-transfection. At 24-hr post-transfection, cell medium was collected from transfected cells, filtered through a 0.45-µm filter, and spun at 25,000 g for 2.5 hr. Pelleted vector-virus was resuspended with a small volume (~0.3% original volume) of medium and aliquots of 0.1 ml/vial were stored at –70°C for future use. Vector-virus was titrated in CEM cells using a serial of 10-fold dilutions. Vector titer was determined at day 3 post-infection by evaluating the GFP expression of infected CEM cell cultures. To generate transduced cells, approximately 2×10^5 HTB cells at exponential growth phase were trypsinized, counted and infected with a concentrated retroviral-vector stock in the presence of 10 µg/ml of polybrene. Following a 1-hr incubation at 37°C, transduced cells were transferred to a TC-25 cm² flask and cultured with fresh medium at 37°C. After ascertaining the transduction efficiency on the basis of GFP expression, the GFP-expressing cells were selected and cloned by two to three cycles of the limiting-dilution cell cloning method.

Viral Plaque Assay

Virus infectivity in sample supernatants was measured by titration in a plaque assay. Briefly, 0.1 ml/well of 10-fold serial dilutions of sample was inoculated onto Vero cell monolayers grown in six-well cell culture plates. After incubation for 1 hr at 37°C with 5% CO₂, unabsorbed residual virus was removed and 3 ml of overlay M199 medium containing 0.5% agar, 350 mg/L of sodium bicarbonate, 29.2 mg/L of L-glutamine, and antibiotics were added to each well. After an additional incubation for 48 hr, 3 ml/well of a second overlay containing 0.5% agar and 0.005% neutral red dye was added to the infected cultures for plaque visualization. The plaques were scored on days 3 and 4 post-inoculation.

Reverse Transcription and Real-Time PCR Analysis of WNV Transcript

At 24 hr post-infection, total RNA was extracted from WNV-infected cells using an RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations. A reverse transcription reaction was carried out using Superscript II Reverse Transcriptase (Gibson, Grand Island, NY), according to the manufacturer's instructions, with a 30 µl reaction mixture

containing 1.5 mg of total RNA. The final volume was brought up to 100 μ l with H₂O for further use. For realtime PCR analysis, 5 μ l of cDNA was amplified in a 25 μ l reaction volume with 200 nM concentrations of each primer using the IQ SYBR GREEN Super mix (Bio-Rad, Hercules, CA). The primers used in this study were described previously [5'-TCAGCGATCTCTCCACCAAAG-3' and 5'-GGGTCAGCACGTTTGTTCATTG-3' [Papin et al., 2004]], and were targeted specifically to the *env* region of the WNV. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (5'-TGGGTGTGAACCATGAGAAG-3' and 5'-GCTAAG-CAGTTGGTGGTGC-3') were used as an internal control for this assay. The reaction was performed in the iQ5 real-time PCR detection system (Bio-Rad).

Following a denaturation step at 94°C for 2 min, 40 cycles of amplification were performed at 94°C for 20 sec and at 60°C for 1 min. Each sample was run in triplicate. Data were analyzed according to the comparative threshold cycle (*C_t*) method, where the amount of target (WNV-*env*), normalized to an endogenous reference (GAPDH) and relative to an experimental control (virus), is given by $2^{-\Delta\Delta C_t}$. *C_t* indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. The ΔC_t value is determined by subtracting the average reference *C_t* value from the average target *C_t* value. The $\Delta\Delta C_t$ value involves subtraction of the ΔC_t experimental control value [Livak and Schmittgen, 2001].

SDS-PAGE and Western Blot Analysis

At 24 hr post-infection, cells were harvested and total cellular proteins were extracted. The protein concentration was determined using the Bio-Rad Protein Assay Kit (Bio-Rad). Extracted cellular proteins were subjected to 10% SDS-PAGE and separated proteins were transferred to PVDF-plus membranes (Osmonics, Westborough, MA) using the Xcell II system (Invitrogen, Carlsbad, CA). Recovered membranes were blotted with primary antibodies (monoclonal anti-WNV envelope antibody 7H2 at dilutions of 1:1,000), followed by incubation with anti-mouse secondary antibody conjugated to horseradish peroxidase (KPL Gaithersburg, MD). Viral proteins were detected by using Super Signal West Pico Chemiluminescence (Pierce, Rockford, IL). The human GAPDH used as a loading control was detected by the monoclonal anti-GAPDH antibody. Visualized bands were measured using the ChemiDoc XRS system (Bio-Rad).

Statistical Analysis

A one-way ANOVA followed by Bonferroni's Multiple Comparison Test (software "Prism") and a *t*-test were employed for statistical analysis in this study. The difference between two samples was considered to be significant if the *P*-value was less than 0.05.

RESULTS

siRNA Decreases WNV Production in Cultured Neuroblastoma HTB-11 Cells

Human neuroblastoma HTB-11 cells were used because WNV is a neurotropic virus that infects HTB cells and induces cytopathic effects (CPE). To study the specific inhibitory effects of retroviral vector-mediated siRNA expression on WNV replication in HTB cells, five siRNA-expressing retroviral vectors targeting the WNV genome were generated. HTB cells stably expressing siRNA were selected by a limited dilution method after cells were infected with retroviral vectors and then with WNV at a multiplicity of infection (MOI) of 1.0. Cell culture supernatant was collected at 48 and 72 hr post-infection and the production of infectious WNV from both transduced and normal control HTB cells were comparatively measured using a viral plaque assay. As shown in Figure 2, virus production from cells expressing siRNA varied compared to the level of virus propagation in control cells. Depending on the siRNA expressed, transduced cells exhibited a varied inhibitory impact on WNV replication. The most efficient

viral inhibition occurred with the use of siRNA targeted at site 312 of the WNV *cap* gene (Fig. 2). In addition, it is notable that the mismatched control siRNA showed no inhibitory effect on virus production.

siRNA Interferes With WNV Replication

The amount of WNV viral RNA transcripts in siRNA-expressing cells was quantified by real-time PCR. HTB cells were infected with siRNA-expressing retrovirus and the cells were then subjected to WNV infection at a MOI of 1. The infected cells were then harvested at 24 hr post-infection and subjected to real-time PCR. The cells expressing siRNA showed a reduction in viral transcripts as compared to that detected in WNV-infected control cells (Fig. 3). Therefore, WNV specific siRNA is capable of reducing the amount of viral RNA transcripts.

To confirm further the inhibitory effects of retroviral vector-mediated siRNA expression on WNV replication, the expression level of viral proteins in transduced cells was assessed. The expression level of the major viral structural envelope (E) glycoprotein (as a representative of the degradation of the entire viral genome) was determined by Western blot assay. As indicated in Figure 4, a decrease in the amount of viral envelope protein was detected at 48 hr post-infection from cells expressing siRNA targeted to the *cap* gene as compared to wild-type WNV-infected cells transduced with the mismatched siRNA or untransduced control cells. Similarly, the concentration of the viral E protein was also significantly lower in siRNA retrovirus-infected cells at 72 hr post-infection as compared to WNV-infected control cells ($P < 0.05$). Although there was an increase in the quantity of viral protein at 72 hr post-infection, the siRNA retrovirus-infected cells still showed lower amounts of viral E proteins. Mock-infected HTB cells, acting as negative controls, yielded no band at these two time points (Fig. 4). This indicated that the anti-sera was able to react specifically with the target viral protein.

DISCUSSION

Successful RNAi producing an inhibition of the viral replication cycle has already been demonstrated in variety of animal viruses such as HIV-1 [Novina et al., 2002; Banerjee et al., 2003], hepatitis C virus [Kapadia et al., 2003; Wilson et al., 2003], hepatitis B virus [McCaffrey et al., 2003; Weinberg et al., 2007], SARS-CoV virus [Zhang et al., 2004], human papillomavirus virus [Hall and Alexander, 2003], and cytomegalovirus [Wiebusch et al., 2004]. The high specificity, versatility, and efficiency of siRNA-mediated inhibition of viral replication suggest its potential use in the therapy and prophylaxis of viral diseases [Carmichael, 2002; Cullen, 2002; Gitlin et al., 2002; Lindenbach and Rice, 2002]. Thus, it is necessary to be able to efficiently deliver and express siRNA in target cells to effectively inhibit virus infections.

This study has demonstrated the first use of retroviral vector-delivered RNAi against WNV virus in cultured human neuroblastoma cells. Standard molecular cloning techniques were employed for the construction and generation of five different siRNA retroviruses, corresponding to different regions of the WNV genome: Capsid, membrane, envelope, and NS5. Capsid, membrane, envelope are structural proteins. NS5 is a non-structural protein, an RNA dependent RNA-polymerase (RdRps). As shown in the results, siRNAs targeting WNV can be efficiently delivered by retroviral vectors and expressed under the control of a *Pol III* promoter H1 in human neuroblastoma cells. WNV production in culture fluid and infected cells, as well as the level of WNV envelope protein, was decreased by retroviral vector-mediated delivery of WNV specific siRNA. Among the siRNA that were tested, only the WNV-specific siRNA targeted at the viral nucleocapsid protein was highly effective, which resulted in a 73% reduction of virus production, whereas other WNV siRNA provided only partial or no inhibition of WNV replication.

These findings are consistent with recent reports that the varied impact of siRNA against WNV replication ranges from no inhibitory effect to almost complete viral inhibition [McCowna et al., 2003; Bai et al., 2005; Geiss et al., 2005; Kumar et al., 2006; Ong et al., 2006]. Ong et al. [2006] reported apparent inhibition of WNV replication by plasmid vectors expressing siRNA targeted at the NS5 gene of WNV. The WNV envelope gene has also been successfully tested as the target gene in recent studies [Kumar et al., 2006; Bai et al., 2005] for of several other sites. Another research group tested multiple sites in the WNV genome and only a few demonstrated limited inhibitory effect [Geiss et al., 2005]. The variation of siRNA-mediated antiviral effect can be attributed to several factors including the level of siRNA expression in the transduced cells and inherent inability of individual siRNA molecules to targeted genes within a viral genome. Since retroviral vector is known to mediate a high level expression of transgenes [Sullenger et al., 1990; Lu et al., 1997; Habu et al., 2005], the selected siRNA molecules themselves may be largely responsible for the moderate level of anti-WNV replication detected in this study.

In addition to the different delivery systems and host cells used in those reports, these experimental results also point toward the importance of the specific sequences within the WNV genome for targeting by the siRNA. The data indicate that there is great variation of sequence-specific RNAi. Therefore, empirical verification of the effectiveness of the designed sequences to suppress target genes is required. The poor inhibition of target sites was probably due to RNA binding to the complex regulatory proteins that are involved in replication, translation or structural stability [Chen et al., 1997; Brinton, 2001; Banerjea et al., 2003]. Moreover, some sequences of the viral mRNA might be buried within secondary structures, or highly folded regions, and may not be easily accessible to interaction with siRNA [Bohula et al., 2003; Kretschmer-Kazemi et al., 2003; Luo and Chang, 2004; Reynolds et al., 2004]. These factors can partly explain the variations in virus suppression observed in this study. In addition, the level of production and stability of siRNA may also affect their interfering abilities [Hohjoh, 2004].

Recent reports have demonstrated successful WNV inhibition by siRNA using lentivirus vectors [Kumar et al., 2006]. In fact, some siRNA have been successfully delivered and expressed by viral vectors, such as retroviruses [Barton and Medzhitov, 2002; Devroe and Silver, 2002], lentiviruses [Abbas-Terki et al., 2002; An et al., 2003; Banerjea et al., 2003], and adenoviruses [Shen et al., 2003; Shen and Reske, 2004] to suppress specific protein synthesis that interferes with virus replication. However, retroviral siRNA systems, a widely used gene therapy tool, have not been reportedly used for the suppression of WNV infection. Results from this study have demonstrated that WNV-specific siRNA can be delivered and expressed effectively by a retroviral vector system and used to suppress WNV virus protein expression and replication. The levels of inhibition of viral protein expression and replication observed in this study were moderate, but similar to those reported in other studies using plasmid vectors, siRNA nucleotides or lentivirus vectors [McCowna et al., 2003; Bai et al., 2005; Geiss et al., 2005; Kumar et al., 2006; Ong et al., 2006].

In conclusion, this study has demonstrated that retrovirus-mediated sequence-specific WNV siRNA expression could be used to interfere with viral protein synthesis and inhibit virus replication, thus suggesting a potential application in the therapy against WNV infection. Results from this and other studies suggest that retroviral-vector expression of siRNA could be a useful tool in addressing the complex interaction of viral and cellular regulatory proteins that are involved in the virus life cycle. Current results argue for studies focusing on testing siRNA sequences targeting other viral genes in order to achieve an improved inhibitory impact on WNV infection.

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Fig. 1.

A: Schematic representation of selected target sites of five siRNA sequences against WNV RNA genome. **B:** A complete human polymerase III H1 transcriptional unit was inserted into the 3'-LTR of murine leukemia virus (MLLV) retroviral vector by using unique *Bgl* II and *Mlu* I restriction endonuclease sites. During reverse transcription, portions of the 3'-LTR serve as the template for generating the new 5'-LTR. The black portion in the U3 region of the 3'-LTR of the schematics represents the unit which expresses siRNA and is copied into the 5' LTR. Ψ , retrovirus packaging signal; H1, human H1 promoter; Ter, transcriptional terminal.

Fig. 2.

WNV replication in siRNA expressing HTB cells. Various siRNA-expressing HTB cells and untransduced control cells were seeded at 5×10^5 cells in tissue culture 25 cm² flasks and then infected with wild type WNV at a MOI of 1.0. Virus replication at 48- and 72-hr post-infection was compared using plaque assay. Mock, HTB cells without WNV infection; mismatch, mutated siRNA (312) against WNV; siRNAs (312, 728, 1005, 9335, 9447) against WNV; Control, WNV-infected and siRNA naive HTB cells. Data are presented as the mean $\pm$ SD (n = 3).

Fig. 3.

Attenuation of WNV mRNA transcripts by siRNA. HTB cells transduced with various retroviral vectors expressing siRNA targeting the WNV genome were infected with WNV at a MOI of 1.0. Cells were harvested at 24 hr p.i., mRNA was extracted and reversely transcribed to cDNA. cDNA was subjected to real-time PCR analysis using gene-specific primers for WNV and GAPDH. The change (n-fold) in WNV mRNA levels are presented as 2^{-Ct} normalized to GAPDH (an endogenous reference) and relative to the virus sample (an experimental control). Control, WNV-infected and –siRNA naïve HTB cells; Mismatch, mutated siRNA 312 against WNV; siRNAs 1005 (envelope) and 312 (capsid) against WNV. Data are presented as means and standard deviation derived from three independent experiments.

Fig. 4. siRNA-mediated reduction of WNV protein expression in WNV-infected HTB cells. HTB cells transduced with various retroviral vectors expressing siRNA targeting the WNV genome were infected with WNV at a multiplicity of infection of 1.0. Total cellular proteins were harvested at 48 and 72 hr post-infection. Western blot analysis was performed using antibody against the WNV envelope protein, or anti-GAPDH antibody as an internal control. Mock (negative control), HTB cells without WNV infection; Mismatch, mutated siRNA 312 against WNV; siRNAs (1005, 312) against WNV; C+ (positive control), WNV-infected and siRNA naive HTB cells. This representative Western blot depicts results obtained from three independent experimental tests.