In vitro and in vivo inhibition of rabies virus replication by RNA interference

Ekaterina A. Durymanova Ono1,2, Keila Iamamoto1,2, Juliana G. Castilho2, Pedro Carnieli Jr.2, Rafael de Novaes Oliveira2, Samira M. Achkar2, Maria L. Carrieri2, Ivanete Kotait2, Paulo E. Brandão1

1Faculdade de Medicina Veterinária, Universidade de São Paulo, São Paulo, SP, Brazil. 2Instituto Pasteur, São Paulo, SP, Brazil.

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Abstract

Rabies is a zoonotic disease that affects all mammals and leads to more than 55,000 human deaths every year, caused by rabies virus (RABV) (Mononegavirales: Rhabdoviridae: Lyssavirus). Currently, human rabies treatment is based on the Milwaukee Protocol which consists on the induction of coma and massive antiviral therapy. The aim of this study was to assess the decrease in the titer of rabies virus both in vitro and in vivo using short-interfering RNAs. To this end, three siRNAs were used with antisense strands complementary to rabies virus nucleoprotein (N) mRNA. BHK-21 cells monolayers were infected with 1000 to 0.1 TCID50 of PV and after 2 hours the cells were transfected with each of tree RNAs in separate using Lipofectamine-2000. All three siRNAs reduced the titer of PV strain in a least 0.72 logTCID50/mL and no cytotoxic effect was observed in the monolayers treated with Lipofectamine-2000. Swiss albino mice infected with 10.000 to 1 LD of PV strain by the intracerebral route were also transfected after two hours of infection with a pool 3 siRNAs with Lipofectamine-2000 by the intracerebral route, resulting in a survival rate of 30% in mice inoculated with 100 LD50, while the same dose led to 100% mortality in untreated animals. Lipofectamine-2000 showed no toxic effect in control mice. These results suggest that intracerebral administration of siRNAs might be an effective antiviral strategy for rabies.

Key words: rabies, RNA interference, siRNAs, antiviral, treatment.

Introduction

Rabies is an acute infectious zoonosis of the central nervous system of mammals caused by Rabies virus (RABV) (Mononegavirales: Rhabdoviridae: Lyssavirus) normally transmitted by the saliva of an infected host, with a fatal outcome (Rupprecht et al., 2002; Acha 2003). The disease has a dramatic clinical course, going from fever, muscle pain and headaches to altered behavior, autonomic dysfunctions and paralysis culminating with death (Butron et al., 2005). RABV has 75 nm in diameter and 150-300 nm in length, with a spiked envelope formed by the G glycoprotein and M matrix protein, with a ribonucleocapsid formed by the L RNA-dependent RNA-polymerase, P phosphoprotein and N nucleoprotein containing the 12 kb negative-sense single-stranded non-segmented RNA (Kaplan 1996; Tordo and Poch, 1998; Wunner 1991). The search for antivirals against rabies is one of the frontiers in the field but, despite a protocol (the Milwaukee Protocol) based on ketamin, ribavirin, midazolam and amantadin was successful after the treatment of a human patient (Wilmoughby et al., 2005), it was shown as not reproducible. RNA interference is an alternative as antiviral technology against RABV already shown as effective in vitro in cell cultures (Brandão et al., 2007; Israsena et al., 2009), but no reports on its in vivo use exist hitherto.

This article reports on the in vitro and in vivo post-transcriptional gene silencing of RABV using three short-interfering RNAs (siRNAs) targeted to the nucleoprotein mRNA using a cationic lipoid as a transfection agent.
Materials and Methods

Reference RABV strain

In vivo and in vitro assays were carried out with the PV strain of RABV grown in BHK-21 cells, with titers of 6.0 log10 TCID50/mL and 7.0 log10 LD50/30 mL (lethal dose by 50%) in BHK-21 and mice, respectively.

siRNAs

The siRNAs described by Brandão et al. (2007) (Table 1), complementary to different regions of RABV N protein mRNA, were synthesized in a duplex format with two deoxy-timidines at the 3’ end of each sense and anti-sense strands and resuspended as instructed by the manufacturers (Invitrogen, Carlsbad, CA, USA) and used for cell and mice trials.

In vitro assays

Each of the three siRNA was tested individually in the in vitro assays. Twenty four-hour-old BHK-21 cells grown in 96 well-plates (one test and one control plate for each RNA, in duplicate) were inoculated with 200 μL of 100 to 0.1 TCID50 of PV in 10-fold dilutions in serum-free minimal essential medium (MEM) from columns 1 to 10 and incubated at 37 °C/5% CO2/2 hours to allow viral penetration (Shankar and Koprowski, 1991), after this incubation period, virus dilutions were discarded and 200 μL of serum-free MEM were added. Next, 100 μL of 1:50 Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) combined with 10 μM of each siRNA in a 1:1 (v/v) proportion, prepared according to manufacturer’s instructions, were added from columns 1 to 10. In columns 11 and 12, 100 μL of serum-free MEM were added. The plates were incubated at 37 °C/5% CO2/22 h to a total 24-hour post-inoculation incubation with at least two RABV replication cycles (Shankar and Koprowski, 1991). Finally, the plates were tested by direct immuno-fluorescence assay (DFA) with anti-RABV nucleocapsid rabbit fluorescein isothiocyanate conjugate (Bio-Rad) according to manufacturer’s instructions and Spearman-Kärber titers were calculated considering as positive wells with at least one fluorescent focus.

In vivo experiment

All in vivo assays in this investigation were conducted with 21 day-old albino-Swiss mice weighing 11 to 14 g, kept at 21 to 25 °C, and food ad libitum and 12 hours with light/day; all animal trials were approved by the Bioethics Committee of the School of Veterinary Medicine, University of São Paulo, Brazil (protocol number 1357/2008). PV virus was diluted from 10,000 to 1 LD50 in 30 μL, inoculated by the intracerebral route by the method described by Koprowski (Koprowski 1996) in 10 mice/dose, divided in treated and control groups. Two hours after inoculation, the treated group was injected by the intracerebral route with 30 μL of an equimolar pool of the three siRNAs combined with 1:1 (v/v) with Lipofectamine 2000, while the control group was mock-treated with 30 μL of serum-free MEM/ Lipofectamine 2000 1:1 combination. Treated and control groups were observed daily during 30 days for rabies signals (seizures, ataxia, hyperesthesia, paralysis and death). The central nervous systems of the dead animals (including those euthanized after 30 days of observation) were tested by DFA as described above. Animals presenting both signals of rabies and a positive DFA result were considered as positives.

Evaluation of Lipofectamine 2000 cytotoxicity in mice

In order to evaluate the cytotoxicity of the transfect on reagent in vivo, five mice were injected intracranially with 30 μL of Lipofectamine 2000 1:50/ serum-free MEM in an 1:1 (v/v) combination, while, five mice (control group) were injected with 30 μL of only serum-free MEM and both groups were observed for 30 days for any clinical manifestations.

Results

In vitro assays

In the in vitro siRNA assay the higher drop in viral titer in the treated plates in comparison with the control

| siRNA | Sequence | Initial position |
|-------|----------|-----------------|
| RNA124 | sense | 5’ GCCUGAGAGAUUAUCUGUGGAG 3’ | 123 |
| | anti-sense | 5’ AUCCACGAUAUUCUGAGGC 3’ |
| RNA750 | sense | 5’ GCACACAGCGUCUCAGCUUC 3’ | 749 |
| | anti-sense | 5’ UAAGCAGUGACAACUGUGC 3’ |
| RNAB | sense | 5’ GACAGCUGUUCCUCACUGUC 3’ | 903 |
| | anti-sense | 5’ AGAGUGAGGAACAGCUGUC 3’ |
plates was found for the siRNAB treatment (Table 2), but siRNAs 124 and 750 caused drops in titers close to that found for siRNAB. For columns 11 and 12 of all treated and control plates, which were not inoculated with RABV and received the Lipofectamine 2000/ serum free MEM, no fluorescent focus was detected and no cytotoxic effect was detected in the monolayers when compared to the control cells (which received only serum-free MEM).

In vivo experiment

In the group of mice inoculated with 100 LD₅₀ and treated with the pool of siRNAs, there was a survival rate of 30%, while, in the control (mock-treated group) inoculated with the same dose, all animals died. With regard to the animals inoculated with 10 LD₅₀, the survival rate among the treated animals and mock-treated animals were 70% and 90%, respectively. For the animals inoculated with 1 LD₅₀, both for the treated and control animals, the survival rate was 100% while, for the groups inoculated with 1,000 and 10,000 LD₅₀, mortality rates were 100% for both treated and control animals. All animals which died after the presentation of rabies tested positive for RABV antigens by the DFA, while all surviving animals (euthanized after 30 days of observation), tested negative.

Evaluation of Lipofectamine 2000 cytotoxicity in mice

All five animals injected intracranially with the Lipofectamine 2000/ serum-free MEM, as well as the other five injected by the same route with only serum-free MEM survived the 30-day observation period with no clinical manifestations or observable health problems.

Discussion

Observing the titers of the PV strain after the treatment of infected BHK-21 cells with the three different siRNAs, the drop in the titer ranged from 0.72 to 0.87 log, with a more intense effect observed for siRNAB. These results demonstrate that all three siRNAs used in this study are similarly efficient when used in separate or as pools, as described by Brandão et al. (2007).

When the three siRNAs were administered as a pool in equimolar concentrations via the intracranial route in mice previously inoculated with the PV strain, a dose-dependent effect between the virus challenge dose and the survival rates was evident, resulting in 30% of survival in mice inoculated with 100 LD₅₀, an indication that the treatment protocol used herein is effective in RABV infections with intermediate doses.

An inverse correlation exists between viral RNA synthesis by transcription and virulence as the accumulation of capsid-binding proteins makes the genomic RNA enclosed in a structure unable support further RNA synthesis (Dietzschold et al., 2008), i.e., the more mRNA for M or N proteins, for instance, the more these proteins are synthesized, encapsidating genomic RNA and making less RNA available for viral synthesis and leading thus to a lower virus titer. Thus, in the case of PV at 50 LD₅₀ in the treated group, it might be speculated that the siRNAs have lowered N mRNA to a level in which RNP encapsidation is diminished and the now nude viral RNA is available to continued RNA synthesis and higher viral titers with a consequent higher virulence, explaining the apparently contradictory results for the higher survival rate of the untreated animals at the same viral dose.

Nonetheless, the influence of the siRNA initial doses must be taken into account, which, in the present study, was 1 and 0.3 nmols in BHK-21 cells and mice, respectively.

Higher doses might be required for a more effective antiviral effect, as shown by Kumar et al. (2006) who used 3.2 nmol of a siRNA against West Nile virus transfected with a cationic lipid six hours after infection in mice and obtained a 100% survival rate. Still, a 200 nmol amount of siRNA, with is 200 times higher than the amount used in BHK-21 cells in the present study, also resulted in an almost 100% survival rate in MK2 cells infected with Monkeypox virus (Alkhalil et al., 2009).

Regarding the Lipofectamine 2000 cytotoxicity assay, BHK-21 cells showed no microscopic alterations after 22 hours and, on the same way, mice injected with Lipofectamine 2000 by the intracranial route showed any signs of toxicity.

The efficiency of cationic lipids as siRNA transfecting agents by the intracranial route in mice, as used in the present study, has been previously described for the Chandipura lethal encephalitis virus (Kumar and Arankalle, 2010), showing that this strategy is validated against neurotropic viruses.

The use of plasmid-delivered pre-micro RNAs transfected with Lipofectamine 2000 also targeted to RABV nucleoprotein mRNA in N2A (mice neuroblastoma) cells was also found efficient to decrease the level of RABV genomic RNA against the CVS strain (Israsena et al., 2009), demonstrating that RABV intracellular cycle can be efficiently inhibited.

However, improved delivery systems are paramount for efficient gene silencing and increases in the time of action and concentration of siRNAs in the cytoplasm can be achieved with plasmids or viral vectors of gene silencing (Aigner 2006).
A further improvement of the delivery of siRNAs to the central nervous system is the conjugation of siRNAs with neurotropic proteins, as reported by Kumar et al. (2007) after the use of a chimeric RABV glycoprotein conjugated with siRNAs against the neurotropic Japanese encephalitis virus, showing a transfection efficiency similar to that obtained with Lipofectamine 2000 when tested in mice and in N2A cells.

In summary, the study presented herein on the use of RNAi against rabies show that this valid antiviral strategy for higher rates of success in rabies treatment protocols in the future.

Ethical approval

All animal trials were approved by the Bioethics Committee of the School of Veterinary Medicine, University of São Paulo, Brazil (protocol number 1357/2008).

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