Isolation of ssDNA aptamers that inhibit rabies virus

Hong-Ru Liang [a,b], Gui-Qiu Hu [b], Tao Zhang [c], Yu-Jiao Yang [a,b], Li-Li Zhao [a,b], Ying-lin Qi [a,b], Hua-Lei Wang [b], Yu-Wei Gao [b], Song-Tao Yang [b,*], Xian-Zhu Xia [b,**]

[a] College of Animal Science and Veterinary Medicine, Jilin University, Changchun 130062, China
[b] Institute of Military Veterinary Medicine, Academy of Military Medical Science, Changchun 130062, China
[c] Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

ARTICLE INFO

ABSTRACT

Aptamers, functional nucleic acids, capable of binding a variety of molecular targets with high affinity and specificity, have emerged as promising therapeutic agents. In this study, the cell surface-systematic evolution of ligands by exponential enrichment (Cell-SELEX) strategy was used to generate DNA aptamers which targeted to the intact rabies virus-infected live cells. Through 35 iterative rounds of selection, five high-affinity single-stranded DNA (ssDNA) aptamers were generated by cell-SELEX. Virus titer assay and real-time quantitative reverse transcription PCR (qRT-PCR) assay revealed that all five aptamers could inhibit replication of rabies virus (RABV) in cultured baby hamster kidney (BHK)-21 cells; and T14 and F34 aptamers were most effective. The qRT-PCR also showed a dose-dependent inhibitory effect in BHK-21 cells. Collectively, these data show the feasibility of generating functionally effective aptamers against rabies virus-infected cells by the Cell-SELEX iterative procedure. These aptamers may prove clinically useful as therapeutic molecules with specific antiviral potential against RABV infections.

© 2012 Published by Elsevier B.V.

1. Introduction

Rabies is a fatal central nervous system (CNS) disease that affects many warm-blooded mammals, typically caused by the rabies virus (RABV). While post-exposure immunization strategies are available to treat RABV-infected patients, they are largely ineffective once the virus has invaded the CNS. Since clinical symptoms of rabies only manifest upon CNS involvement, which can occur 1 to 3 months after infection, most patients are incurable and the mortality rate is nearly 100% [1]. As one of the worldwide human health threats, rabies causes an estimated 60,000 deaths annually [2]. Hence, there is an urgent need to identify and develop alternative therapeutic strategies to treat RABV infections.

An individual's ability to successfully clear the infection of many pathogenic diseases is often determined by the outcome of the race between the development of effective immune response and pathogen spread. In particular, when rabies virus spreads faster than an effective anti-virus immune response can be developed, it may overwhelm the host immune system, leading to death in the acute phase of the infection. Thus, immunotherapeutic intervention that decreases production of virus particles in the infected cells will likely curb the spread of the virus [3].

Aptamers are single-stranded RNA or DNA molecules evolved in vitro to specifically recognize and tightly bind cognate targets by means of well-defined secondary and 3-dimensional structures [4],

which are generated by an iterative in vitro process called systematic evolution of ligands by exponential enrichment (SELEX). Not only do aptamers combine the advantages of antibodies, such as high affinity, excellent specificity and low toxicity or immunogenicity, but also they are stable and easy to synthesize, modify and manipulate [5].

The therapeutic potential of aptamers has been demonstrated by several studies, and aptamers have been introduced into clinical use. For example, a study on currently available HIV-1 entry inhibitors revealed that RNA aptamers elicited the most potent in vitro antiviral efficacy, and suppressed viral replication by up to 10,000-fold [6]. Furthermore, an aptamer-based anti-VEGF treatment for age-related macular degeneration, Macugen (pegaptanib sodium injection), which was developed by Pfizer, was approved by the United States Food and Drug Administration in 2004 as sufficiently safe and effective for human treatment.

In this study, we aimed to generate ssDNA aptamers with therapeutic potential against RABV infection. The Cell-SELEX iterative method was used to create a panel of aptamers that bound to rabies virus-infected live cells and reduced viral replication in an in vitro infection model.

2. Materials and methods

2.1. Cells, viruses, and reagents

Baby hamster kidney (BHK)-21 cells were purchased from the Chinese Institute of Veterinary Drug Control (Beijing, China). The cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum

* Corresponding author. Tel.: +86 431 86985808; fax: +86 431 8985888.
** Corresponding author. Tel.: +86 431 86985922; fax: +86 431 8985869.
E-mail addresses: yst610223@yahoo.com.cn (S.-T. Yang), Xia_xzh@yahoo.com.cn (X.-Z. Xia).

(FBS; Hyclone, Logan City, UT, USA) and 100 units/mL of penicillin–streptomycin (PAA Laboratories, Pasching, Austria).

RABV strains: CVS-11, ERA, HEP-Flury and SRV9 strains were used for this study and obtained from the Changchun Institute of Veterinary Science (Changchun, China). Virus samples were grown in BHK-21 cells in DMEM containing 2% FBS, and stored at −80 °C until use.

2.2. Virus growth in cells

To determine the growth curve of virus in BHK-21 cells, monolayer cultures of 1×10^7 BHK-21 cells were infected with the CVS-11 strain at various multiplicity of infection (MOI) values, including 0.01, 0.1, 0.5, 1.0, and 2.5, and incubated at 37 °C. At 24 h, 48 h, 72 h, 96 h and 120 h post-infection (p.i.), a 100 μL aliquot of culture supernatant was removed to determine viral titer using the fluorescent antibody test (FAT), according to the manufacturer's instructions. To further observe viral growth, monolayers of BHK-21 cells in 96-well plates were infected with serial 10-fold dilutions of virus suspension and incubated for 4 days at 37 °C. Cells were then fixed with 80% acetone for 1 h at 4 °C and stained with fluorescein isothiocyanate (FITC)-labeled antibody against N protein of RABV (Fujirabio). Antigen-positive foci were counted under a fluorescent microscope (CKX41; Olympus, Tokyo, Japan) and calculated as focus forming unit (FFU) per mL.

2.3. Cell-SELEX primers and library

Aptamers were selected from a 45-mer randomized library, which had the following 18-mer primer overhang sequences at the 5′- and 3′-ends respectively [7]: 5′-ATCCAGAGTGACGCAGCA-3′ and 5′-ACTAAGCCACCGTGTCCA-3′. The sense-strand of the primers was labeled at the 5′-end with FITC to facilitate flow cytometry-based monitoring of the progress of selection. The antisense-strand of the primers was labeled at the 5′-end with biotin After denaturing in alkaline conditions (0.15 M NaOH), the FITC-conjugated sense-strands were separated from the biotin-conjugated antisense strands by using streptavidin-coated Sepharose beads (Promega Corp., Madison, WI, USA). The corresponding ssDNA pool was then applied to iterative-round selection.

2.4. Cell-SELEX procedure

BHK-21 cells were grown to 70% confluency in 60 mm dishes and infected with CVS-11 at an MOI of 0.5 for 48 h at 4 °C. For aptamer selection, a pool of ssDNA (10 nmol) was dissolved in 1 mL of binding buffer (0.1 g/L yeast tRNA (Invitrogen, NY, USA), 4.5 g/L glucose, 1.0 g/L bovine serum albumin (Solarbio, Beijing, China), and 5 mmol/L $MgCl_2$ in Dullbecco's PBS (Invitrogen, NY, USA)) and denatured by heating at 95 °C for 5 min. Samples were cooled on ice for 10 min before being added to the rabies virus-infected BHK-21 cells, which had been replated on a 60 mm diameter cell culture dish (Corning Inc., Corning, NY, USA). The mixture was incubated with shaking (60 rpm) for 60 min at 4 °C. The supernatant was then removed and the cells were washed with at least 5 mL wash buffer (4.5 g/L glucose and 5 mmol/L $MgCl_2$ in Dullbecco's PBS). The cells were then harvested and transferred into 500 μL DNase-free water. The cell surface-bound ssDNAs were eluted by heating at 95 °C for 10 min, and harvested by centrifugation at 12,000 rpm for 5 min. Then, the 100 μL ssDNA pool was used as template of the following PCR reaction mix: 100 μL 10× PCR buffer, 80 μL dNTPs (2.5 mM each; Takara Bio Inc., Dalian, China), 50 μL FITC- and biotin-primer mixture (0.5 μM final concentration), 660 μL DNase-free water, and 10 μL *rTaq* DNA polymerase (Takara Bio Inc. Dalian, China). The thermal cycling amplification conditions included: one denaturation cycle at 94 °C for 3 min, followed by 16 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, and a final extension cycle at 72 °C for 7 min.

To remove non-specific bounding DNAs, we applied a counter-selection step using uninfected BHK-21 cells followed by the selection of infected cells in each cycle. To improve the affinity and specificity of selected aptamers, we increased the stringency of the above-described selection procedure by: 1) increasing washing times from 3 to 5; 2) increasing the volume of wash buffer from 2 mL to 10 mL; 3) increasing the volume of FBS from 10% to 20%; 4) serially reducing the cell number; and 5) gradually increasing the incubation temperature from 4 °C to 37 °C.

2.5. Cloning and sequencing of DNA aptamers

After 35 rounds of selection, the selected ssDNA pool was PCR-amplified using unmodified primers. The final PCR products were purified using the MinElute PCR purification kit (Qiagen, Germantown, MD, USA). The purified products were ligated into the pEASY-T1 vector using the accompanying Simple Cloning kit (TransGen Biotech, Beijing, China) and following the manufacturer's instructions. Isolated individual clones were analyzed by sequencing and aptamers were synthesized.

2.6. Flow cytometric analysis

FITC-labeled aptamer pools or individual aptamers were generated by PCR with FITC-labeled primers. The FITC-conjugated sense-strands of ssDNA were separated from the biotin labeled antisense-strands as described above. To evaluate the efficiency of enrichment of aptamer candidates during selection, infected BHK-21 cells were treated with trypsin for 1–2 min and then cultured in 1 mL DMEM rocking (60 rpm) for 2 h at 37 °C to recover the surface proteins. Then, FITC-labeled aptamers (250 nM) were mixed with 10^6 infected cells in 500 μL binding buffer (0.1 g/L yeast tRNA (Invitrogen, NY, USA), 4.5 g/L glucose, 1.0 g/L bovine serum albumin (Solarbio, Beijing, China) and 5 mmol/L $MgCl_2$ in Dullbecco's PBS (Invitrogen, NY, USA)) and incubated with shaking (60 rpm) for 60 min at 4 °C. The cells were harvested by centrifugation at 2000 rpm for 5 min, and washed twice with 2 mL wash buffer. Finally, the cells were resuspended in 500 μL binding buffer for analysis by flow cytometry. The initial library was used as a control for all samples. The stained cells, which were tightly bound by FITC-conjugated ssDNA, were then measured with a FACScan flow cytometer (Becton Dickenson, Franklin Lakes, NJ, USA) by counting 30,000 events and results were analyzed by FlowJo software.

2.7. Binding experiments

FITC-labeled aptamers at various concentrations in binding buffer were incubated with the infected BHK-21 cells for 60 min at 4 °C. After washing three times, cells were resuspended in 500 μL binding buffer for analysis by a FACScan flow cytometer (Becton Dickenson, Franklin Lakes, NJ, USA). For binding curves, values of nonspecific binding of the controls were subtracted from the aptamer binding values. The apparent dissociation constants (Kd) of the aptamer-cell was determined by SigmaPlot according to the equation: $Y = B_{max}X/(Kd + X)$.

2.8. Virus titer assay

In order to investigate whether the aptamers were able to inhibit CVS-11 production, two BHK-21 infection models were generated. In the first model, CVS-11 (MOI = 0.5) and different aptamers (300 nM) were added simultaneously to BHK-21 cells. In the second model, BHK-21 cells were infected with CVS-11 for 24 h, and then exposed to different aptamers (300 nM). For each of the infection models, the cells were incubated at 37 °C for 48 h after aptamer addition. The virus titers were measured using the FAT test. Results from at least three independent assays for each aptamer were averaged.