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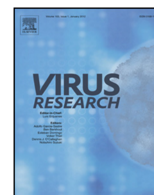

Selection of an aptamer against rabies virus: A new class of molecules with antiviral activity

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ABSTRACT

Rabies is a fatal central nervous system (CNS) disease caused by the neurotropic rabies virus (RABV). The therapeutic management of RABV infections is still problematic, and novel antiviral strategies are urgently required. We established the RVG-BHK-21 cell line, which expresses RABV glycoprotein on the cell surface, to select aptamers. Through 28 iterative rounds of selection, single-stranded DNA (ssDNA) aptamers were generated by exponential enrichment (SELEX). A virus titer assay and a real-time quantitative reverse transcription PCR (qRT-PCR) assay revealed that four aptamers could inhibit the replication of RABV in cultured baby hamster kidney (BHK)-21 cells. However, the aptamers did not inhibit the replication of other virus, e.g., canine distemper virus (CDV) and canine parvovirus (CPV). In addition, the GE54 aptamer was found to effectively protect mice against lethal RABV challenge. After inoculation with aptamers for 24 h or 48 h, followed by inoculation with CVS-11, approximately 25–33% of the mice survived. In summary, we selected aptamers that could significantly protect from a lethal dose of RABV in vitro and in vivo.

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1. Introduction

Rabies is one of the oldest recognized fatal infectious diseases. The causative agent of the disease is rabies virus (RABV). Data obtained from global mortality estimates suggest that every 10 min, one person dies from rabies, and approximately 300 others are exposed to rabies (Allendorf et al., 2012). Rabies is a viral infection of the CNS that is almost always fatal once symptoms occur. Although rabies has been known since the Middle Ages, basically no effective treatment for the disease is available once symptoms occur, resulting in more than 50,000 deaths worldwide per year (Albertini et al., 2008).

RABV is an unsegmented negative-sense RNA virus that contains five genes (the N, P, M, G and L genes) encoding nucleoprotein (N protein), phosphoprotein (P protein), matrix (M) protein, glycoprotein (G protein) and large (L) protein (Masatani et al., 2011). G protein is the only viral protein that is glycosylated and exposed at the surface of the virion. This protein is responsible for interaction with receptors to enter target cells (Yamada et al., 2012). Aptamers are oligonucleotide molecules that bind to a specific target molecule. Aptamers fold into unique tertiary conformations that can bind to target antigens with a high affinity and specificity, analogous to antibodies (Farokhzad et al., 2006). Aptamers, which are typically single-stranded nucleic acids, have high potential to be powerful tools for diagnosis and therapy because of the following advantages over antibodies: low antigenicity, easy and reproducible synthesis, easy modification and low toxicity (Kunii et al., 2011).

Aptamers themselves are considered to be promising candidates for many therapeutic applications and have also been used to facilitate the targeted uptake of other therapeutic molecules (Chen et al., 2008). An aptamer-based anti-vascular endothelial growth factor (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 (Liang et al., 2012). In the present study, we established the RVG-BHK-21 cell line, which expressed RABV glycoprotein on the cell surface. We performed a SELEX procedure for the isolation of aptamers that targeted RVG-BHK-21 cells and could efficiently inhibit RABV replication. These aptamers can effectively inhibit RABV both in vitro and in vivo.

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2. Materials and methods

2.1. Cells, viruses, animals and reagents

Baby hamster kidney (BHK)-21 cells, feline kidney (F81) cells and African green monkey kidney (Vero) cells, purchased from the Chinese Institute of Veterinary Drug Control (Beijing, China), were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FBS; HyClone, Logan City, UT, USA) and 100 units/ml penicillin–streptomycin (PAA Laboratories, Pasching, Austria). The RABV standard strain CVS-11, canine distemper virus (CDV), canine parvovirus (CPV) and the pCAGGS/Neo plasmid were obtained from the Changchun Institute of Veterinary Science (Changchun, China). Four-week-old female BALB/c mice (weighing 20–22 g) were obtained from the Changchun Institute of Biological Products, Changchun, China.

The wash buffer contained 4.5 g/l glucose and 5 mmol/l $MgCl_2$ in Dulbecco's PBS (Invitrogen, NY, USA). The binding buffer used for selection was prepared by adding yeast tRNA (Invitrogen, NY, USA) and 1.0 g/l bovine serum albumin (Solarbio, Beijing, China) to the wash buffer.

2.2. Establishment of RVG-BHK-21 cell line

The G gene from the RABV CSV-11 strain was amplified by PCR (GF: GC CTCGAGGCCGCCACCATGGTTCCTCAGGTTC, *XhoI* underlined; GR: GCAGATCTTCA CTTGTACAGCTCGTCCATGCCGAGA, *BglII* underlined) and inserted into the *XHoI* and *BglII* restriction sites of pCAGGS/Neo to construct the plasmid pCA-G, which contained a neomycin resistance (neo) gene. The expressed protein was located in the cell membrane by a signal peptidase of pCAGGS/Neo. BHK-21 cells were transfected with the plasmid pCA-G using Lipofectamine 2000 (Invitrogen Life Technologies). For the colony formation assay, cells were grown in regular medium for 2 days, followed by medium containing G418 geneticin (Invitrogen Life Technologies; 550 μg/ml) for 14 days. G418-resistant colonies were pooled and named RVG-BHK-21 cells.

To analyze the cell surface expression of RABV glycoprotein by indirect immunofluorescent staining, RVG-BHK-21 cells were cultured in 96-well plates for 24 h and then fixed in 80% acetone. The cells were incubated with mouse anti-rabies antibody (1:200), followed by FITC-conjugated goat anti-mouse IgG (Sanjian, China) (1:500). The surface expression of RABV glycoprotein was determined using a fluorescent microscope (CKX41; Olympus, Tokyo, Japan).

2.3. SELEX procedure

Aptamers were selected from a 45-mer randomized library, which were flanked by two conserved sequences for PCR amplification and commercially synthesized by Invitrogen (Shanghai, China). The synthetic ssDNA library (10 nmol), dissolved in 1 ml binding buffer, was pre-incubated for 5 min at 95 °C and then for 10 min at 4 °C. The library was then added to RVG-BHK-21 cells, which had been replated on a cell culture dish (Corning Inc., Corning, NY, USA). The mixture was incubated for 60 min at 4 °C, and the cells were then washed with at least 5 ml wash buffer. Binding sequences were amplified by PCR to generate a new pool. To remove nonspecifically bound DNAs, we applied a counter-selection step using BHK-21 cells, followed by the selection of RVG-BHK-21 cells, in each cycle and an increase in the incubation temperature from 4 °C to 37 °C.

After 28 rounds of selection, the resulting DNA was cloned 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. Later, specific aptamers were synthesized.

2.4. Flow cytometric analysis

To evaluate the enrichment of aptamer candidates during selection, aptamer pools or individual aptamers were generated by PCR using FITC-labeled primers and incubated with cells for 60 min at 4 °C. The cells were then resuspended in 500 μl binding buffer for analysis using flow cytometry. The initial library was used as a control for all samples. The stained cells, which were tightly bound to FITC-conjugated ssDNA, were then measured with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) by counting 30,000 events, and the results were analyzed by FlowJo software.

2.5. Virus titer assay

To evaluate whether the aptamers could inhibit CVS-11 production, RABV-infected BHK-21 cells were divided into three groups: (i) CVS-11 (MOI = 0.5) and the various aptamers (300 nM) were added simultaneously to BHK-21 cells; (ii) after BHK-21 cells were infected with CVS-11 (MOI = 0.5) for 24 h, the different aptamers (300 nM) were added to the medium; and (iii) BHK-21 cells were infected with CVS-11 (MOI = 0.5) after being incubated with the aptamers for 24 h. In all cases, when CVS-11 and the aptamers were added to the medium, the cells were incubated at 37 °C for another 48 h, and the virus titers were measured by the Reed–Muench method.

2.6. Aptamers against different virus-infected cells

To investigate whether the aptamers could specifically inhibit RABV production, Vero cells were infected by CDV, F81 cells were infected by CPV, and BHK-21 cells were infected by RABV. Various aptamers (300 nM) were added simultaneously. The cytopathic effects (CPEs) of CDV and CPV were examined under a light microscope, and the virus titers of individual samples were calculated by the Reed–Muench method. The results of at least three independent assays for each aptamer were averaged.

2.7. Characterization of aptamers

FITC-labeled aptamers at various concentrations in binding buffer were incubated with the RABV-infected BHK-21 cells for 60 min at 4 °C. After washing three times, the cells were resuspended in 500 μl binding buffer for analysis by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For binding curves, the values of the nonspecific binding of the controls were subtracted from the aptamers' binding values. The apparent dissociation constant (K_d) of the aptamer-cell binding was determined by SigmaPlot according to the equation $Y = B_{max}X/(K_d + X)$. Y represents the average fluorescence intensity; B_{max} represents the maximum fluorescence intensity measured; K_d is the dissociation constant; X is the concentration of aptamer added.

Moreover, structural analysis of the aptamers was performed to predict the most likely minimum energy structures by MFOLD (http://mfold.rna.albany.edu/?q=mfold/DNA-Folding-Form).

2.8. MTT assays

BHK-21 cells were plated onto a 96-well plate and incubated with aptamer from 10 μM to 0 μM at 37 °C for 48 h. Moreover, cells were plated onto a 96-well plate in the presence or absence of aptamers (from 10 μM to 0 μM) at 37 °C for 48 h. The viability assay was performed by adding 20 μl/well MTT solution (Takara Bio Inc.,

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