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Development of potential antiviral strategy against coxsackievirus B4

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ABSTRACT

Coxsackievirus B4 (CVB4) can cause a broad range of diseases such as aseptic meningitis, meningoencephalitis, myocarditis, hepatitis, pancreatitis, gastroenteritis, necrotizing enterocolitis, pneumonia and sudden death in the neonates. CVB4 has also been implicated as a possible etiological agent for type 1 insulin dependent diabetes mellitus (IDDM). In this study, the possibility of RNA interference (RNAi) as a potential therapeutic approach to treat CVB4 infection was explored. The results showed that the Rhabdomyosarcoma (RD) cells treated with 19-mer siRNAs displayed high specificity against CVB4 replication without displaying any sign of target effects. The siRNA targeting the $3C^{pro}$ region of CVB4 genome was also established to be the most effective in inhibition of CVB4 replication in RD cell line in a dosage dependent manner, indicating its potential to be developed as an antiviral strategy against CVB4.

1. Introduction

Coxsackievirus B4 (CVB4) is a member of the *Enterovirus* family and is one of the six serotypes of coxsackievirus group B (CVB) (Reuckert, 1990). Like all CVBs, CVB4 has been known to elicit a wide variety of diseases in the world. The most common result of general CVB infection is asymptomatic infection, undifferentiated febrile illness, or mild upper respiratory symptoms (Pallansch, 1997). Severe disease symptoms are readily recognised by the presence of fever, chest pain, pleural inflammation, headache and sore throat. Infections by CVB4 have also been known to cause aseptic meningitis, encephalitis, pleurodynia, myocarditis, and pericarditis (Crowell and Landau, 1997). Although most of the diseases caused by CVB4 are also commonly seen in infections with other enteroviruses, nevertheless, clinical symptoms such as myopericarditis and pleurodynia (Bornholm disease) are still distinct and are associated only with CVB4 infections (Modlin and Rotbart, 1997). However, the most significant chronic disease associated with CVB4 infection is the juvenile onset of insulin dependent diabetes mellitus (IDDM) (Pallansch, 1997). The ability of CVB4 to induce glucose intolerance, resulting in viral-induced insulin dependent diabetes mellitus (IDDM) has long been suspected and demonstrated both in vitro (Yoon et al., 1979; D'Alessio, 1992; Fohlman and Firman, 1993) and in vivo (Yoon et al., 1986; Horwitz et al., 1998; Richardson et al., 2009).

There are currently no available vaccines for non-polio enteroviruses (Pallansch, 1997) and no antiviral therapy for enterovirus infections. By far, the only available treatment is directed towards alleviating the symptomatic effects as a result of CVB4 infections. There has been successful identification of candidate drugs exhibiting antiviral activity against enteroviruses in tissue culture and animals (Rozhon et al., 2005). Several of this class of drugs are currently in development and in early clinical trials (McKinlay and Steinberg, 1986; O'Connell et al., 1995). However, they have not been evaluated in the treatment of diseases in animal model systems of both myocarditis and diabetes (Ilback et al., 1993; See and Tilles, 1993). Hence, there is a need for an antiviral therapy which is specific for treating CVB4 infections. RNA interference (RNAi) is an evolutionary conserved mechanism that has been observed across all eukaryotic cells (Dykxhoorn et al., 2006). RNAi plays an important role in the endogenous silencing of repetitive or transposable genetic elements for the maintenance of cellular homeostasis (Siijen and Plasterk, 2003). RNAi has also been shown to confer protection to human cells from infections caused by viruses by inhibiting the transcription of viral transcripts (Gitlin et al., 2002; Lecellier et al., 2005). In this study, we evaluated RNAi as potential antiviral strategy against CVB4 in an in vitro system.

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2. Materials and methods

2.1. Virus strains

The ATCC CVB4 J.V.B strain (Accession number X05690) which was used in this study, was provided by Department of Microbiology, National University of Singapore for a research project undertaken by APY Wong during his undergraduate course.

2.2. Design of chemically synthesized 19-mer small-interfering RNAs (siRNAs)

The siRNAs were designed to target the 2A, 3C and 3D regions which encode for non-structural genes of the CVB4 genome ($2A^{pro}$, $3C^{pro}$ and $3D^{pol}$) based on the general guidelines described by Elbashir et al. (2001). Using the cluster alignment function of the DNASTAR program, highly conserved regions within the three regions were identified and entered into the siRNA Target Finder software (Ambion, Inc.). Successfully identified siRNA targets were then subjected to analysis by the BLAST algorithm. Non-specific scrambled sequence of the siRNA targeting different regions of $3D^{pol}$ region was designed and used as a control. All siRNAs were synthesised by Sigma Proligo, USA (Table 1).

2.3. Transfection and infection

Rhabdomyoscarcoma (RD) cells were maintained in growth medium (MEM supplemented with 5% FCS; Gibco, USA), 2% HEPES and 1.5% $NaHCO_3$. When the growth of the RD cells reached confluence, the cell monolayer was rinsed with phosphate buffered saline (PBS) to remove dead cell and debris before treatment with 1X trypsin-EDTA to detach the cells. Upon detachment of the RD cells from the bottom of the flask, fresh growth medium was added to stop the reaction. The cells were then passaged at a ratio of 1:4 in T75 tissue culture flasks and maintained in a 37 °C incubator with 5% CO_2. For RNAi studies, RD cells were seeded at a 5×10^4 cells/well concentration into a 24-wells microtiter plate. Transfection of RD cells with siRNAs was based on a serum starvation protocol using Lipofectamine™ 2000CD (Invitrogen, USA). The transfection protocol was optimised according to manufacturer's instructions. The growth medium was replaced with 500 μl of OPTI-MEM® (Gibco, USA) 24 h after the cells were seeded into the microtiter plate. After 24 h of starvation with OPTI-MEM®, the RD cells were transfected with a transfection mixture containing 1.2 μl of Lipofectamine™ 2000CD diluted in OPTI-MEM® containing different concentrations of siRNAs (0, 25, 50 and 100 nM). The transfection mixtures were added into individual wells and incubated for 48 h. Mock transfection was carried out without the addition of siRNAs as the negative control. At 48 h post-transfection, the transfected RD cells were infected with CVB4 at 30 plaque forming units (pfu) for 1 h. At different time points post-infection, cell supernatant was first collected. The RD cells were then lysed using CellLytic buffer (Invitrogen, USA) and centrifuged for the cell lysate. Both the cell lysates and cell supernatants were stored at −80 °C for further studies.

Table 1
Nucleotide sequences of the 19-mer siRNAs.

siRNA	Region	Nucleotide sequence
siRNA_2A	2A	5′-GAG AGT GAG TAT TAC CCC A TT-3′ (Sense) 5′-TGG GTA AAT ACT CAC TCT C TT-3′ (Anti-sense)
siRNA_3C	3C	5′-GCT GTG CTC GCC ATT AAC A TT-3′ (Sense) 5′-TGT TAA TGG CGA GCA CAG C TT-3′ (Anti-sense)
siRNA_3D	3D	5′-ATT GAA GGA ATG TAT GGA C TT-3′ (Sense) 5′-GTC CAT ACA TTC CTT CAA T TT-3′ (Anti-sense)
siRNA_SC	Scrambled	5′-GGA ATT TAT GAA ATG CGG A TT-3′ (Sense) 5′-TCC GCA TTT CAT AAA TTC C TT-3′ (Anti-sense)
siRNA_3DF	3D	5′-(Fluo)ATT GAA GGA ATG TAT GGA C TT-3′ (Sense) 5′-GTC CAT ACA TTC CTT CAA T TT-3′ (Anti-sense)

2.4. Observation of transfection efficiency

RD cells were seeded onto Labteck Permanox® Chamberslide (Nunc, Germany) and transfected with varying concentrations (0, 10, 50 and 100 nM) of the fluorescein labelled siRNA (siRNA_3DF). As a control, one of wells was treated with 100 nM of siRNA_3DF without Lipofectamine™ 2000CD. At 48 h post-transfection, the cells were washed twice with PBS and fixed with methanol for 10 min. The cells were washed again with PBS before addition of Vectashield Mounting Medium with propidium iodide (Vectorlabs, USA). The cells were then observed using the Olympus BX60 fluorescent microscope.

2.5. Cell viability assay

Cell viability assay was carried out to determine cell viability using the CellTiter 96® AQueous Non-radioactive Cell Proliferative Assay (Promega, USA). The MTS/PMS reagent of the assay is composed of a tetrazolium compound that is reduced by dehydrogenases within viable RD cells into a formazan product. Briefly, at 48 h post-transfection, the RD cells were trypsinized and transferred into 96-well microtiter plate in triple aliquots of 100 μl. Subsequently, 20 μl of MTS/PMS reagent were added into each 100 μl aliquot of cells. After incubation at 37 °C for 3 h in the dark, the absorbance of formazan was measured at OD 490 nm. Hence, the amount of formazan formed is directly proportional to the number of viable cells.

2.6. Plaque assay

To determine the viral infectivity of CVB4 after siRNA treatment, plaque assays were carried out after the transfection protocol. In brief, 48 h after infection with 30 pfu (plaque forming units) of CVB4, the plaque media was removed and the RD cells fixed with 7.5% of paraformaldehyde. Once the cells were fixed, the plaques were visualised by staining the RD cells with 1% crystal violet for half an hour at room temperature.

2.7. Real-time RT-PCR

At 48 h post-infection, the RD cells were harvested and total viral RNA extraction was carried out by addition of 150 μl of CellLytic™ M Cell Lysis Reagent (Sigma, USA) into the individual wells containing the RD cells. Viral RNA was then extracted using the QIAmp® Viral RNA Mini Kit according to the manufacturer's instructions (Qiagen, USA).

The efficiency of the various siRNAs in inhibiting CVB4 replication were then analysed using the real-time TaqMan RT-PCR assay. A pair of primers (namely CVB_3DF and CVB4_3DR) and a TaqMan probe was designed from the highly conserved regions within the 3D region (Table 2). The real-time RT-PCR TaqMan assay was carried out using the LightCycler® (Roche® Molecular Biochemicals, Germany). The LightCycler® machine achieves efficient heat transfer to the amplification mixture through the use of air currents and glass capillary reaction vessels. The sensitive fluorescent detectors allow real-time monitoring of amplification process through changes in emission of fluorescence.

The LightCycler® RNA Amplification Hybridization Probes kit (Roche® Molecular Biochemicals, Germany) were used in this study. The test kit allows a one-step RT-PCR in glass capillaries using the LightCycler instrument. The enzyme mix contains a mixture of