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ScienceDirect
Antiviral Research 74 (2007) 36–42
Antiviral Research
www.elsevier.com/locate/antiviral

Effective inhibition of porcine transmissible gastroenteritis virus replication in ST cells by shRNAs targeting RNA-dependent RNA polymerase gene

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Received 22 June 2006; accepted 29 December 2006

Abstract

Transmissible gastroenteritis virus (TGEV) is identified as one of the most important pathogenic agents during swine enteric infection, leading to high mortality in neonatal pigs and severe annual economic loss in swine-producing areas. Up to date, various vaccines developed against TGEV still need to be improved. To exploit the possibility of using RNA interference (RNAi) as a strategy against TGEV infection, two shRNA-expressing plasmids (pEGFP-U6/P1 and pEGFP-U6/P2) targeting the RNA-dependent RNA polymerase (RdRp) gene of TGEV were constructed and transfected into swine testicular (ST) cells. The cytopathic effect (CPE) and MTS assays demonstrated that both shRNAs were capable of protecting cells against TGEV invasion with very high specificity and efficiency. A real-time quantitative RT-PCR further confirmed that the amounts of viral RNAs in cell cultures pre-transfected with the two plasmids were reduced by 95.2% and up to 100%, respectively. Our results suggest that RNAi might be a promising new strategy against TGEV infection.

Keywords: TGEV; RNA interference (RNAi); Short hairpin RNA (shRNA); RdRp; ST

1. Introduction

Transmissible gastroenteritis virus (TGEV), the causative agent of porcine transmissible gastroenteritis (TGE), at all ages of pigs results in gastroenteritis, characterized by vomiting, yellowish diarrhea and dehydration. The consequences of TGE vary among different ages of pigs. Suckling piglets often suffer a mortality of as high as 100% (Saif and Wesley, 1999; Kim and Chae, 2001), while older animals generally show growth retardation due to high morbidity, both of which result in enormous economic loss in swine-producing areas in the world every year (Chen and Schifferli, 2003; Schwegmann-Wessels and Herrler, 2006; Sestak et al., 1996).

As a member of the *Coronaviridae*, TGEV has a genome of positive, single-stranded RNA. The genomic RNA has a length of 28.5 kb. Approximately 5′ two-thirds of the genome (from the 5′-end) comprises open reading frames (ORF) 1a and 1b, mainly encoding the RNA-dependent RNA polymerase (RdRp), whereas the one-third from the 3′-end is mainly made up of the genes encoding viral structural proteins, such as spike (S), envelope (E), membrane (M) and nucleocapsid (N) protein (Alonso et al., 2002; Escors et al., 2003; Penzes et al., 2001). The genome itself may also serve as mRNA, together with other six subgenomic mRNAs transcribed discontinuously from the negative-strand template, forming a nested set of RNAs of different lengths with co-terminal ends (Alonso et al., 2002; Lai and Cavanagh, 1997; Laude et al., 1990).

Vaccines have been considered as the best control of TGEV infections (Ho et al., 2005; Saif, 1996; Torres et al., 1995; Wesley and Lager, 2003). However, many vaccine strategies developed so far have not been entirely successful. Firstly, inactivated TGEV vaccines generally offer poor protection in swine, while the attenuated ones are not ideal in their safety due to the risk of reverting to a virulent form, and some mucosal vaccines may even induce adverse reactions (Holmgren et al., 2003; Opriessnig et al., 2002; Saif and Wesley, 1999; Wesley and

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doi:10.1016/j.antiviral.2006.12.007

Table 1
The inserted sequences in shRNA-expressing plasmids

plasmids	inserts
pEGFP-U6/P1	5'-GTACTGGGATCGCACATATTTCAAGACGATATGTGCGATCCCAGTACTTTTTT-3'
pEGFP-U6/P2	5'-GGCAAGAGCTCGTACAGTATTCAAGACGTACTGTACGAGCTCTTGCCTTTTTT-3'
pEGFP-U6/T	5'-GACTTCATAAGGCGCATGCTTCAAGACGGCATGCGCCTTATGAAGTCTTTTTT-3'

Lager, 2003). Furthermore, neonatal piglets may suffer from gastroenteritis within 20 h post-infection and death may happen in 1–4 days (Schwegmann-Wessels and Herrler, 2006), whereas current vaccines cannot provide complete protection prior to 7 days after inoculation (Brim et al., 1995; de los Santos et al., 2005). So, it is urgently needed to develop a high-effective, rapid-acting antiviral strategy against TGEV. On the other hand, current research on antiviral protection triggered by RNA interference (RNAi) in vivo mainly employed mice as experimental models (Liu et al., 2006; Palliser et al., 2006). However, studies of candidate therapeutic strategies using an adult mouse model of subclinical enteric viral infections often do not predict their efficacy in neonatal large mammals (Yuan and Saif, 2002).

RNAi is a biological process that functions as an ancient defense mechanism of cells against hostile genes and regulates the function of normal genes during growth and development (Arenz and Schepers, 2003; Bagasra and Prilliman, 2004; Ratcliff et al., 1997; Zambon et al., 2006). Since it was found that experimental introduction of exogenous double-stranded RNA into *C. elegans* and mammalian cells could specifically silence homologous mRNA (Fire et al., 1998; Elbashir et al., 2001), RNAi has been the most fascinating technique for hostile gene knockdown and viral inhibition (Borkhardt and Heidenreich, 2004; Robson and Sacks, 2005; van Rij and Andino, 2006). Within a few years, a wide variety of disease-associated viruses have been targeted effectively throughout plant, animal and human kingdom, such as severe acute respiratory syndrome coronavirus (SARS-CoV), human immunodeficiency virus (HIV), influenza virus and hepatitis viruses (Huelsmann et al., 2006; Li et al., 2005; Shlomai and Shaul, 2004; van Rij and Andino, 2006; Wu et al., 2005). Here, we report the first study to investigate whether shRNA-mediated RNA interference could inhibit TGEV replication in swine testicular (ST) cells.

2. Materials and methods

2.1. Cell culture, virus propagation and titration

Prior to being challenged with TGEV TGEs-1 strain, ST cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% heat-inactivated super neonatal bovine serum (NBS), 5% L-glutamine and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) in a 37 °C, 5% CO_2 incubator overnight. After 70% of the virus-infected cells showed cytopathic effects (CPE), the cultures were collected for three frozen–thawed cycles, and then serially diluted by 10-fold. Each dilution was added to ST cells in four-fold. After 3 days of infection, the viral 50% cell culture infectious dose ($CCID_{50}$) was calculated using the Reed and Muench method.

2.2. ShRNA sequences selection and expression plasmids construction

The RdRp gene of TGEV is well conserved among different TGEV strains and was used as the RNAi target in this study. Lots of sequences homologous to the RdRp coding region of TGEV SC-Y strain (GenBank accession no.: DQ443743) were generated by the web site siRNA designing tools (http://www.ambion.com/techlib/misc/siRNA_finder and http://www.oligoengine.com/Home/mid_prodSirna.html_sirna_tool), from which two theoretically effective sequences at nt positions 13160–13180 (P1) and 13949–13969 (P2) within the open reading frame 1b were selected. To guarantee a similar RNAi effect on different TGEV strains, the two sequences were analyzed by a BLAST search in the GenBank nucleotide database to avoid any similar sequence found in the swine genome, but share a 100% homology within the published sequences of different TGEV strains. To investigate the specificity of viral inhibition, a non-specific sequence (T) also underwent a BLAST analysis and served as a negative control. Their corresponding sequences are separately shown in Table 1. To investigate whether shRNA-mediated RNAi could block TGEV infection in ST cells, all the three sequences were generated to make shRNA-expressing plasmids: pEGFP-U6/P1, pEGFP-U6/P2 and pEGFP-U6/T, respectively. The three inserted sequences were arranged as the following alignment: *Bam*HI+ Sense+ Loop+ Antisense+ Termination signal+ *Sal*I+ *Hin*dIII (Table 1). Enhanced green fluorescence protein (EGFP) gene fused in the plasmids was used as a reporter during the transfection efficiency analysis. The RNA transcripts from these U6 promoter-containing plasmids were expected to fold back and form a stem-loop structure with a 19-base pair region homologous to the RdRp gene of TGEV.

2.3. Cell transfection and virus infection

One day before transfection, ST cells were seeded in 48-well plates at a density of 2–3 × 10^4 cells/well without antibiotics. When the cells reached 70–80% confluency, the medium was