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Specific small interfering RNAs-mediated inhibition of replication of porcine encephalomyocarditis virus in BHK-21 cells

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Abstract

Encephalomyocarditis virus (EMCV) is recognized as a pathogen inducing acute myocarditis and sudden death in preweaned piglets and severe reproductive failure in sows. In this study, eight specific small interfering RNA (siRNA) duplexes targeting different genomic regions of EMCV BJC3 were designed and their ability to inhibit virus replication in BHK-21 cells was investigated. The results showed that BHK-21 cells transfected with siRNA duplexes to 2C gene (JH-4666, BJC-1739), 2B gene (BJC-807), 3C gene (BJC-2363) and 3D gene (BJC-3269) were specifically resistant to EMCV infection when exposed to 500 times the 50% cell culture infective dose ($CCID_{50}$) of EMCV. The levels of the 3D gene in the transfected cells were obviously decreased. IFA and Western blotting analysis confirmed that the expression of VP1 protein in cell culture transfected with the siRNAs was apparently reduced. Of the five siRNAs, JH-4666, BJC-2363 and BJC-3269 were the most effective. Combination of the siRNA duplexes enhanced the inhibition of EMCV replication. Our data indicated that specific siRNAs are able to inhibit the replication of porcine encephalomyocarditis virus in BHK-21 cells, suggesting that RNAi might provide a new approach to prevent EMCV infection.

Keywords: Encephalomyocarditis virus (EMCV); RNA interference (RNAi); Small interfering RNA (siRNA); EMCV BJC3 isolate; BHK-21 cells; Inhibition; Replication

1. Introduction

Encephalomyocarditis virus (EMCV) is a member of the genus Cardiovirus, family Picornaviridae. The genome is a single-stranded, positive-sense RNA of approximately 7.8 kb with a unique large open reading frame (ORF) (Palmenberg et al., 1984). Similar to other picornaviruses, the genome of EMCV is covalently linked at the 5′ end to a protein called virion protein, genome-linked (VPg) (Flanegan et al., 1977), in the 5′untranslated region (UTR) it contains the internal ribosomal entry site (IRES). The 3′UTR ends with a short heterogeneous poly(A) tail. Among the genes of EMCV, the 3D gene that encodes the RNA-dependent RNA polymerase is the most conserved. The genomic RNA serves as a template for viral RNA transcription to synthesize more copies of positive genomic RNA through a negative intermediate, and also acts as an mRNA template for translation of a single polyprotein that is post-translationally processed primarily by proteases 2A and 3C to produce individual structural proteins including VP1, VP2, VP3 and VP4 protein and nonstructural proteins.

Since fatal disease of swine caused by EMCV was first described in 1958 (Murnane et al., 1960), the virus has been recognized worldwide as a pathogen that can infect many host species including pigs, rodents, cattle, elephants, raccoons, marsupials, and primates such as baboons, monkeys, chimpanzees (Gelmetti et al., 2006; Koenen, 2006; LaRue et al., 2003). Some evidences of EMCV infection and seroprevalences in humans imply that the virus imposes potential significance in public health (Deutz et al., 2003a,b; Juncker-Voss et al., 2004; Kirkland et al., 1989). The pig is considered the most commonly

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and severely infected domestic animal species (Acland and Littlejohns, 1975; Billinis et al., 1999). EMCV infection could cause acute myocarditis and sudden death in preweaned piglets (Billinis et al., 1999; Gelmetti et al., 2006) and severe reproductive failure in sows (Dea et al., 1991; Koenen et al., 1994; Love and Grewal, 1986), resulting in severer economic losses for swine production (Acland and Littlejohns, 1975; Koenen et al., 1999). Recently, EMCV infection in pigs was shown to be prevalent in many intensive swine farms (J.L. Zhang et al., 2007), and EMCV isolates have been identified and characterized in China (Ge et al., 2007; G.Q. Zhang et al., 2007). Moreover, EMCV may be a potential pathogen for recipients in xenotransplantation (Brewer et al., 2003; Denis et al., 2006). Xenotransplantation research has focused on using pig tissues and cells, including heart valves, skin, hepatocytes and neural cells to treat various diseases in humans (Lanza and Cooper, 1998). Porcine islet cells (PICs), which are susceptible to porcine EMCV, are used in clinical trials for treatment of type I diabetes in humans. Although EMCV infection does not appear to affect insulin production by PICs, infected xenografts can transmit the virus to recipient animals, resulting in severe disease (Brewer et al., 2004). Recently, transplantation of myocardial and pancreatic tissues from acutely infected pigs transmitted the virus to recipient mice, resulting in acute fatal EMCV disease, suggesting that the virus may provide a risk in pig to human transplantation (Brewer et al., 2003). Therefore, preventing EMCV infection in pigs is very important for swine production and future xenotransplantation in human.

Vaccination is considered as one of the effective strategies for controlling EMCV infection. Although traditional and novel vaccines associated with EMCV have been reported (Hunter et al., 1998; Osorio et al., 1996; Sin et al., 1997; Suh et al., 2001), these vaccines have not been applied widely in practice. Moreover, it has been shown that the vaccine was effective in pigs as early as 7 days post-exposure (Osorio et al., 1996), but did not provide early protection against EMCV infection in piglets. Thus, developing a new rapid-acting and effective antiviral strategy against EMCV infection in piglets will be worthy to be considered. Recent studies have shown that RNA interference (RNAi) is active and effective against infection of animal pathogenic viruses (Chen et al., 2006; He et al., 2007; Huang et al., 2006; Kahana et al., 2004; Liu et al., 2005; H. Zhou et al., 2007; J.F. Zhou et al., 2007). These data support the use of RNAi as a novel antiviral therapy in animals. In this study, we investigated the ability of short interfering RNAs (siRNAs)-mediated RNA interference for EMCV replication in baby hamster kidney (BHK) cells by using siRNAs targeting different regions of the genomic RNA of EMCV.

2. Materials and methods

2.1. Cell cultures and viruses stock

Baby hamster kidney cells (BHK-21) and MARC-145 cells (China Institute of Veterinary Drug control) were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO^{TM}, Invitrogen Corporation) supplemented with 10% (v/v) bovine calf serum (GIBCO^{TM}, Invitrogen Corporation), 10 mM Hepes, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin (pH 7.4). BHK-21 cells were used for propagation and challenge of EMCV. EMCV BJC3 was isolated and identified in our laboratory (Ge et al., 2007; G.Q. Zhang et al., 2007). Porcine reproductive and respiratory syndrome virus (PRRSV) BJ-4 strain (North American type) was characterized in our laboratory (Yang et al., 1997) was used as control virus in this study. Determination of the virus titers was conducted by a standard 50% cell culture infective doses ($CCID_{50}$) assay using the Reed and Muench method (Reed and Muench, 1938).

2.2. Selection and preparation of siRNAs

According to the genomic sequences of EMCV BJC3 (GenBank accession no. DQ464062), eight siRNAs corresponding to viral capsid protein 1A and 1C, viral protease 2A and 3C, viral nonstructural protein 2B and 2C, and RNA-dependent RNA polymerase 3D were designed by Ambion's siRNA "Target Finder and Design Tool" available at http://www.ambion.com/techlib/misc/siRNA_finder. Specificity of these sequences was verified by BLAST search of the National Center for Biotechnology Information's expressed sequence tag library. All siRNA duplexes consisted of two complementary 21-nucleotide RNA strand with 3′dTdT overhangs and were chemically synthesized (Shanghai GenePharma Co. Ltd.). Scrambled siRNA duplex (Shanghai GenePharma Co. Ltd.) with no sequence similarity to the genome of EMCV was used as negative control to ensure the specificity of the observed effects. The sequences of the siRNAs used in the current studies are summarized in Table 1.

2.3. Transfection of siRNAs and virus infection

BHK-21 cells were trypsinized and seeded in 12-well plates (Costar, Corning Incorporated, U.S.A.) at 4×10^5 cells per well in DMEM containing 2% bovine calf serum 1 day before transfection and were maintained in a 5% CO_2 humidified incubator at 37 °C. Then, the cells were transfected under optimal conditions. Briefly, when the cells reached about 70–80% confluence, they were washed and overlaid with transfection complexes containing 2 μg chemically synthesized siRNA duplexes in 100 μl of OPTI-MEM medium (GIBCO^{TM}) mixed with DMRIE-C Reagent (Invitrogen Corporation) according to the Manufacturer's instructions. A mock control (the cells transfected by transfection reagent with no siRNA) was used to check for any cytotoxicity arising from the reagent, and FITC negative control siRNA duplex was used to check the transfection efficiency by BD FACSCalibur^{TM} (Becton, Dickinson and Company). All 12-well plates were filled with 1 ml medium per well. After 4 h post-transfection, the cells were infected with 500 $CCID_{50}$ of EMCV BJC3 per well. After incubation at 37 °C, 5% CO_2 humidified incubator for 30 min, the cells were washed twice and cultured with DMEM containing 2% bovine calf serum. Cells were examined with an Olympus microscope (Olympus, U-LH 50HG, Japan), and images were collected with an Olym-