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In vitro inhibition of Japanese encephalitis virus replication by capsid-targeted virus inactivation

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ABSTRACT

Japanese encephalitis virus (JEV) is a leading member of the mosquito-transmitted flavivirus family, and is mainly distributed in China, India and South East Asia, where it can cause the central nervous system disease with irreversible neurological damage in humans and animals. Few effective anti viral drugs are currently available against JEV infections. To explore the feasibility of using capsid-targeted viral inactivation (CTVI), as an anti viral strategy against JEV infection, a plasmid pcDNA-Cap-SNase was constructed for expressing a fusion protein of JEV capsid (Cap) and *Staphylococcus aureus* nuclease (SNase). Under G418 selection, a mammalian cell line BHK-21/Cap-SNase stably expressing Cap-SNase fusion proteins could be detected by rabbit antiserum against JEV and had good nuclease activity in degrading DNA or RNA. The viral titer from JEV-infected BHK-21/Cap-SNase cell line was reduced about 69.7% compared with that produced in control BHK-21 cells. It was clearly demonstrated that Cap-SNase fusion proteins could be use to efficiently inhibit JEV replication, resulting in a reduction of viral titer. Therefore, the CTVI approach might be applicable to JEV inhibition as a novel anti viral strategy.

1. Introduction

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* within the family *Flaviviridae*. Members of the genus *Flavivirus* are predominantly arthropod-borne viruses, such as dengue virus (DEN), West Nile virus (WNV), yellow fever virus (YFV), and tick-borne encephalitis virus, and frequently cause significant morbidity and mortality in mammals and birds (Hills and Phillips, 2009; Misra and Kalita, 2010). And there are 30,000–50,000 cases of human Japanese encephalitis worldwide and 10,000–15,000 deaths each year. By some estimates, there may be as many as 75,000 cases each year (Saxena et al., 2009). JEV has been expanding its 'geographical footprint' into previously non-endemic regions and with several billion people at risk, Japanese encephalitis (JE) represents an internationally emerging concern in tropical and sub-tropical countries (Nazmi et al., 2010). The JEV genome is approximately 11 kb in length that carries a single long open reading frame (ORF) flanked by a 95-nt 59 untranslated region (59 UTR) and a 585-nt 39 UTR. The ORF encodes a polyprotein which is processed by viral and cellular proteases into three structural and seven non structural proteins (Sumiyoshi et al., 1987; Vrati et al., 1999).

At present, a live attenuated JEV vaccine (SA14-14-2 strain) with excellent immunogenicity is widely used in humans and pigs in China and other countries in Asia (Yu, 2010; Gao et al., 2010). But there is still no specific therapeutic approaches available for JEV-positive humans and animals. Therefore, it is urgent to develop a new and effective anti viral strategy against JEV infection. A protein-based anti viral strategy called capsid-targeted viral inactivation (CTVI), in which virion structural protein-nuclease fusion proteins are targeted to virions, where they inactivate the virus by degrading its genomic RNA (Natsoulis and Boeke, 1991; Schumann et al., 1996). In this strategy, the viral capsid protein is designed as the carrier of a deleterious enzyme, such as a nuclease, a proteinase, or even a single-chain antibody to bind to a native viral protein (Okui et al., 2000). Expression of this fusion protein in chronically infected cell culture resulted in its incorporation into virions and subsequent inactivation of the virus particles by degradation of viral RNA. Release of particles incorporating Cap-SNase fusion proteins into the extracellular milieu activates the nuclease and results in destruction of the virion from within. CTVI has been extensively investigated and showed to be a promising anti viral strategy against a few important viruses, such as murine leukemia virus (MLV) (Natsoulis et al., 1995; Schumann et al., 2001; VanBrocklin et al., 1997; VanBrocklin and Federspiel, 2000), human hepatitis B virus (HBV) (Liu et al., 2003) and human immunodeficiency virus (HIV) (Kobinger et al., 1998; Beterams and Nassal, 2001), CTVI was also found to have anti viral effect in other members of the Flaviviridae family such as Dengue virus (Qin et al.,

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2003; Qin and Qin, 2004, 2006) and classical swine fever virus (Zhou et al., 2010; Wang et al., 2010).

Although the capsid protein has very little amino acid homology among flaviviruses, for example, the homologies of the capsid protein of JEV to those of WNV, DEN type 2 (DEN2), and TBEV were only 67%, 33%, and 25%, respectively, the structural properties, such as the hydrophobicity profile, abundance of basic amino acid residues, and secondary and tertiary structures, are well conserved (Dokland et al., 2004; Christopher et al., 2003; Ma et al., 2004). We have fused *Staphylococcus* nuclease (SNase) to C-terminal end of JEV capsid protein as part of a general strategy for destroying flaviviruses from within. The SNase anti viral moiety was chosen for its size and basic biochemical properties. During assembly, there should be sufficient space within the virion for the SNase protein to be incorporated. Most importantly, however, SNase has a strict requirement for calcium (0.5–1 mM for optimal activity). In mammalian cells, intracellular concentrations of calcium are typically in the nanomolar range, preventing cellular nucleic acids from being degraded. Sera and other extracellular body fluids, in contrast, contain millimolar concentrations of calcium. Therefore, SNase is presumably active extracellularly (Boeke and Hahn, 1996) and may be an appropriate candidate for CTVI.

In the present study, it was successfully proved that CTVI could be used against JEV infection with Cap-SNase fusion proteins stably expressed in BHK-21 cells. Cap-SNase fusion proteins were not cytotoxic to host cells, and infection of the BHK-21/Cap-SNase stable cell line with the JEV NJ-2008 strain showed that SNase could be functionally incorporated into progeny JEV virions where it effectively inhibited the subsequent spread of JEV by plaque reduction assay, Q-PCR and western blot analysis at 96 h post-infection. These results suggest that CTVI could be a new anti-JEV strategy.

2. Materials and methods

2.1. Construction of the pcDNA-Cap-SNase expression vector

For construction of the vector expressing the fusion protein, a pair of specific primers (Cap1: 5′-GGGGTACCATGACTAAAAAAC-CAGGA-3′; Cap2: 5′-AATGGATCCGCTCCTGCACAAGCTAT-3′) were designed and used to amplify the coding region of the JEV Cap. The forward and reverse primers contained the restriction sites (underlined) for *Kpn* I and *BamH* I, respectively. The primers were synthesized by Invitrogen (Shanghai Co., China), and the predicted PCR product was 386 bp. Viral RNA was extracted from cell cultures of JEV NJ-2008 strain (GQ918133) using TRIzol reagent (Invitrogen, CA, USA). To generate cDNA, 100 ng of RNA was used for each reaction. The reaction mixtures, containing 13 μl total RNA, 1 μl random primers, 1 μl of 10 mM dNTPs and 4 μl 5 × RT buffer, were incubated at 65 °C for 10 min followed by incubation on ice for 5 min, and further incubation at 37 °C for 1 h after adding 1 μl of moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen). The subsequent PCR was performed using high-fidelity DNA polymerase (Takara, Dalian, China). The PCR conditions were 94 °C for 5 min for pre-denaturing, then 35 cycles for denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. The SNase gene was amplified by using a pair of primers (SNase-P1: 5′-GAAGGATCCAACAGTATATAGTGC-3′; SNase-P2: 5′-GCCGAATTCT-TATTGACCTGAATCAGCG-3′) from eukaryotic expression vector pcDNA-NP-SNase (a gift from Prof. Ping Jiang). The forward and reverse primers contained the restriction sites (underlined) of BamH I and *EcoR* I, respectively. The PCR consisted of 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. The PCR products (469 bp) were separated by electrophoresis in a 1% agarose gel followed by ethidium bromide staining. Then the gene Cap and SNase were ligated into the expression plasmid pcDNA3.1/v5-His and resulted in pcDNA-Cap-SNase plasmid (Fig. 1). The positive recombinant products were confirmed by restriction enzyme digestion and DNA sequencing.

2.2. Cell transfection

Twenty-four hours before transfection, 2×10^6 BHK-21 cells (ATCC CCL-10), cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37 °C in the presence of 5% CO_2, were seeded into each well of a 6 well plate (Corning). The medium was replaced with serum-free DMEM 1 h before transfection. BHK-21 cells were transfected with 4 μg of pcDNA-Cap-SNase or 4 μg of blank plasmid pcDNA3.1/V5-His and 10 μl of Lipofectamine™.

2000 Reagent (Invitrogen) according to the manufacturer's protocol. At the same time, non transfected BHK-21 cells were used as the negative control. After incubation for 48 h, the medium was replaced with DMEM media containing G418 (GBICO, CA, USA) at a final concentration of 800 μg/ml for resistance screening. Based on the color of the medium and cell growth, the medium was changed every 2–3 d. When a large number of cells died, selection was maintained with 600 μg/ml G418. After 4–6 weeks, the cells were cultured in G418-free medium. The cells with good growth condition were selected for further amplification. Cells were harvested for analysis of transgene expression, and culture supernatants were collected to detect Cap-SNase fusion protein expression.

2.3. Identification of the expressed Cap-SNase fusion protein

2.3.1. Rt-pcr

Total RNA was isolated from the transfected BHK-21 cells cracked using TRIzol reagent. The RNA was treated with DNase, and transcription of the Cap-SNase gene from the recombinant plasmid in BHK-21 cells was detected by RT-PCR. The Cap gene was detected by primers Cap1 and Cap2 as described above, and the SNase gene was detected using a pair of primers SN-P1 and SN-P2 as described above.

2.3.2. Western blot analysis

Total cellular extracts were obtained for western blot analysis by lysis of cells in cell lysis buffer (Boshide Biotech Co, Wuhan, China). Protein concentrations of the cell lysates were determined by

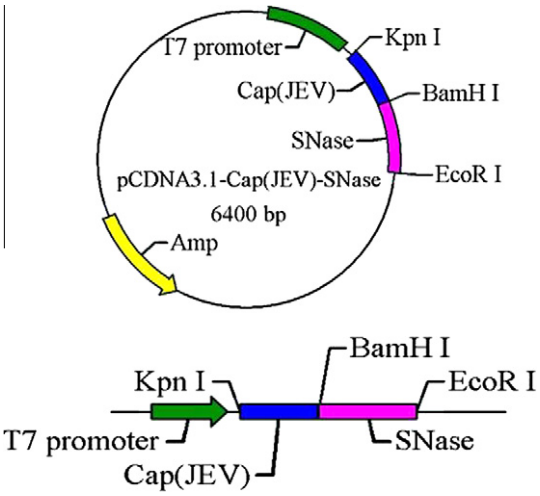

Fig. 1. The structure of the protein expression vector, constructed in pcDNA-3.1/V5-His plasmid. pcDNA-Cap-SNase contains the gene Cap and SNase by the restriction sites (Kpn I, BamH I and EcoR I).