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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

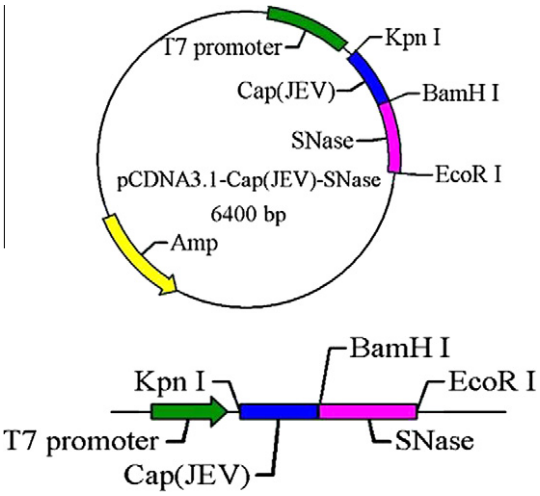

Fig. 1. The structure of the protein expression vector, constructed in pcDNA-3.1/V5-His plasmid. pcDNA-Cap-SNase contains the gene Cap and SNase by the restriction sites (Kpn I, BamH I and EcoR I).

the Coomassie blue dye-binding assay (Bio-Rad, CA, USA). An equal volume of samples were boiled for 5 min and proteins were separated by 12% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane (Millipore, MA, USA) by a semi-dry transfer method (Maniatis et al., 1989). The membranes were blocked with TBST buffer (10 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween-20) containing 5% skim milk over night, then incubated with rabbit anti-JEV antibody (1:50) for 2 h, and followed by the addition of goat anti rabbit IgG (Santa Cruze) (1:1000) for 1 h and detection was performed using chemilumines (Zhang et al., 2011).

2.3.3. Ifa

The normal BHK-21 cells and the BHK-21/Cap-SNase cells which were under G418 were put in 6 well plate for 12 h, until they grow to cell monolayers, respectively. Then the cells were fixed in acetone for 15 min at −20 °C and washed three times with PBS (pH 7.4). Then rabbit anti-JEV antibody (1:100) was applied for 1.5 h at 37 °C. After washing three times with PBS for 5 min, respectively, the cells were incubated with FITC-goat anti rabbit IgG (Pierce) at a dilution of 1:1000 for half an hour at 37 °C. The stained cells were observed under fluorescence microscopy (Olympus BX-51, Olympus Co., Tokyo, Japan) that was equipped with a digital camera.

2.4. Nuclease activity assays

In vitro nucleic acid digestion assays were conducted as described previously (Qin et al., 2005; Qin and Qin, 2006). Briefly, 1×10^6 transfected cells were harvested and re-suspended in 0.5 ml cell lysis buffer, followed by centrifugation at 1000*g* for 10 min. The supernatant was collected and boiled for 5 min and 100 ng pcDNA-3.1/V5-His plasmid DNA or total RNA from JEV-infected BHK-21 cells extracted with TRIzol reagent was added and incubated in the reaction solution (100 mmol/L Tris–HCl pH 8.8, 10 mmol/L $CaCl_2$) at 37 °C for 30 min. The reaction was stopped by adding 0.15 mol/L EDTA. The efficacy of the recombinant nuclease was detected by 1% agarose gel electrophoresis, and nuclease activity was determined by monitoring nucleic acid disappearance.

2.5. Plaque reduction assay

The cell line was continuously passaged for 15 generations or more under G418 selection, which was named as BHK-21/Cap-SNase cells. The compounds showed inhibition of virus replication in the CPE inhibition assay, which were further evaluated using plaque reduction assay. Briefly, BHK-21 (2×10^6 cells/well) cells were grown to a confluent monolayer in a 6 well plate and then infected with 0.1 MOI JEV for 1.5 h at 37 °C. At the end of adsorption, the supernatant of each well was removed, and then overlaid with 1.5 ml of 1% molten agarose (Sigma–Aldrich, USA) in DMEM containing 2% FBS, and the plates were incubated for 4 days at 37 °C, appropriate controls were included in each run of the assay. At the end of incubation period monolayers were fixed with formalin, then the agarose was gently removed and the cells were stained using 1% crystal violet. Three independent observers counted the plaques using a hand lens. All the experiments were run in triplicates. Percentage inhibitions of plaques were determined using the formula given below:

$$\text{Inhibitions}\% = (1 - \text{mean PFU}_{\text{BHK-21/Cap-SNase}}/\text{mean PFU}_{\text{BHK-21}})^{*}100\%$$

2.6. Real-time PCR

To quantify JEV RNA in BHK-21 cells and BHK-21/Cap-SNase cells, real-time PCR was carried out using a pair of primers for amplifying the JEV gene. At 24, 48, 72, 96 h post-infection (hpi), viral RNA was extracted from each well by using TRIzol reagent. RNA pellets were suspended in 25 μl DEPC-treated water and a reverse transcription (RT) reaction was performed by utilizing an RT reaction kit (Takara, Dalian, China). Two pairs of specific primers were designed to amplify a 323 bp fragment to JEV and 97 bp fragment to BHK-21 cell in the conserved by utilizing the DNAMan software, respectively (JEV1F: 5′-CCCTCAGAACCGTCTCGGAA-3′; JEV2R:5′-CTATTCCCAGGTGTCAATATGCTGT-3′; BHK1F: 5′-CCCCAA TGTGTCCGTCGTG-3′, BHK2R:5′-GCCTGCTTCACCACCTTCT-3′), real-time PCR was performed by using SYBR Green PCR master mix (Takara, Dalian, China), 0.25 mM each primer and 20 μl of the RT reaction, following the manufacturer's protocol (Applied Biosystems). Samples were heated for 30 s at 95 °C and a two-step cycle (5 s at 94 °C, 31 s at 60 °C was repeated 40 cycles. These two segments were cloned into pMD-18T vector to construct the standard plasmid, respectively, used to obtain a relative standard curve. Relative quantification of JEV RNA relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control. The $2^{-\triangle\triangle Ct}$ method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments (Livak and Schmittgen, 2001). The results of $2^{-\triangle\triangle Ct}$ showed the difference between experimental group and control group. $\triangle\triangle Ct$ = BHK-21/Cap-SNase ($Ct_{JEV} - Ct_{\beta\text{-actin}}$)-control BHK-21 ($Ct_{JEV} - Ct_{\beta\text{-actin}}$).

2.7. Anti viral activity

BHK-21 cells and BHK-21/Cap-SNase cells were infected similarly as described above for the relative quantitative RT-PCR. To quantify JEV E protein in BHK-21 cells and BHK-21/Cap-SNase cells, western blot analysis was carried out to investigate the anti viral property of CTVI. At the indicated time points, the cells were lysed in 100 μl cell lysis buffer with 5% of β-mercaptoethanol, and boiled for 10 min. The protein concentration was determined by the Brad ford method. Briefly, 15 μg of protein from each sample was separated by 12% SDS–PAGE and transferred by electroblotting onto nitrocellulose membrane using semi-dry transfer cell according to the manufacturer's manual. The membrane was then treated sequentially with blocking solution (PBST containing 5% non-fat skim milk), then probed with anti-JEV E mAb 4D1 (a gift from Prof. Sheng-bo Cao, Huazhong Agricultural University, Wuhan, China) (Li et al., 2010), or anti-actin antibody (Santa Cruz) as described above. Bound antibody was detected using peroxidase-conjugated secondary antibodies (Cell Signaling), and visualized using enhanced chemiluminescence (ECL; GE Healthcare Life Sciences).

2.8. Statistical analysis

All assays described here were repeated at least three times, and all the measurements were made in triplicate. Mean values ± standard deviation (SD) were calculated using SPSS biostatistics software (version 16.0, SPSS Inc., Chicago, IL, USA). Statistical analysis was done by one way analysis of variance and values were considered significant or when $p < 0.05$. Figures were performed using the Graph Pad TM Prism 5.0 software.

3. Results

3.1. Stable expression of Cap-Snase fusion protein in BHK-21

The recombinant plasmid pcDNA-Cap-SNase was digested with kpnI, BamHI and EcoRI, respectively. The results showed that Cap gene and SNase gene were in the correct reading frame after being inserted into eukaryotic expression vector pcDNA3.1/v5-His pro-

ven by restriction enzyme digestion and DNA sequencing. Then the recombinant plasmid pcDNA-Cap-SNase was transfected into the BHK-21 cells. The cell line was passaged continuously for 15 generations or more under G418 selection, which was named as BHK-21/Cap-SNase cells. In order to confirm whether the screened BHK-21/Cap-SNase cells expressed stably Cap gene (386 bp) and SNase gene (469 bp), PCR was performed by using specific primers to amplify the products from the isolated total RNA (Fig. 2, **lanes 2 and 4**); however, the products could not be detected in Rnase-treated total RNA (negative control) (Fig. 2, **lanes 3 and 5**), indicating that fusion gene Cap-SNase was transcribed into the BHK-21/Cap-SNase cells. To further identify the expression products of the recombinant plasmid, Western blot was carried out using rabbit anti-JEV antibody. A protein band of 31 kDa (Cap = 14 kDa and SNase = 17 kDa) was detected in the transfected cell lysis (Fig. 3). The molecular weight was calculated online by using ExPASy protemics at the server (http://www.expasy.ch/tools/pi_tool.html). The expressed fusion proteins were detected by indirect immunofluorescent signals in the cytoplasm (Fig. 4), indicating that the BHK-21/Cap-SNase cell line was stable after being passaged continuously for 15 generations.

3.2. Analysis of nuclease activity of Cap-Snase fusion protein

In order to verify whether the SNase of the fusion proteins retained biological activity, in vitro nucleic acid digestion assays were designed to determine nuclease activity. The cell lysates obtained after boiling of the BHK-21/Cap-SNase cells effectively degraded plasmid DNA or RNA in a buffer with 10 mmol/L Ca^{2+} concentration. The activity of 5 μl of cell lysates containing Cap-SNase was similar to that of 0.8 pg of a standard preparation of SNase (Shengxing Co. Lot, Nanjing, China) in vitro DNA digestion assay (Fig. 5A), while the activity of 7.5 μl of cell lysates containing Cap-SNase was similar to that of 0.8 pg of a standard preparation of SNase (Fig. 5B). The results showed that plasmid DNA, virus 42S RNAs (Takeda et al., 1977) and ribosomal RNAs (28S and 18S) could all be digested with Cap-SNase fusion proteins of cell line lysates. This nuclease degraded both RNA and DNA and, more importantly, depended on millimolar Ca^{2+} concentrations for activity.

3.3. Anti viral efficacy of BHK-21/Cap-SNase

3.3.1. 1Real-time PCR to test JEV mRNA

The ability of inhibiting JEV replication in BHK-21/Cap-SNase cell line was determined by real-time PCR. BHK-21/Cap-SNase cells were inoculated with viruses at 0.1 MOI for 1.5 h at 37 °C and the normal BHK-21 cells with same amount of JEV were used as the positive control. The results of real-time PCR showed that at 48 hpi, the expression of JEV mRNA of BHK-21/Cap-SNase cell line were dramatically decreased compared with that of JEV-infected BHK-21 cells (Fig. 6, p value < 0.05). However, at 72 and 96 hpi, it were more significantly less, respectively (Fig. 6, p value < 0.01). This illustrated that the BHK-21/Cap-SNase cell line had the exactly anti viral activity in vitro.

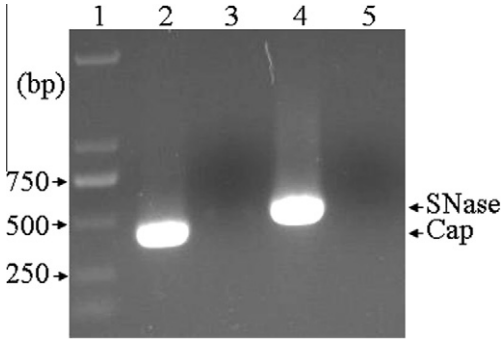

Fig. 2. RT-PCR analysis of mRNA in BHK-21/Cap-SNase cells. Lane 1: DL 2000 Marker; Lanes 2, 4: RT-PCR of the Cap and SNase the genes, respectively, from total RNA; Lanes 3, 5: RT-PCR of the Cap and SNase the gene, respectively, from total RNA treated with RNase.

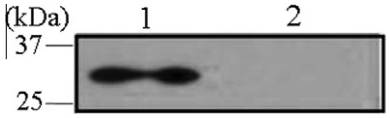

Fig. 3. Analysis of Cap-SNase fusion proteins by Western blot. Lane1: BHK-21/Cap-SNase cells; Lane 2: BHK-21 cells control.

3.3.2. Reduction of JEV viral load in vitro due to BHK-21/Cap-SNase cell line

To assess whether the BHK-21/Cap-SNase cell line had any effect on reduction of viral load in vitro, the BHK-21/Cap-SNase cell line was subjected to plaque assay as described above (Fig. 7A). The viral titer from JEV-infected BHK-21/Cap-SNase cell line was significantly reduced compared with those from JEV-infected BHK-21 cells, which was reduced about 69.7% ((1-50750/167500)*100%) (Fig. 7B, p value < 0.01). To further validate the results obtained from the plaque assay, western blot analysis was performed. The results showed that the JEV viral load was significantly reduced in both 48, 72 and 96 hpi (Fig. 8) due to the reduction of E protein. These result indicated that the BHK-21/Cap-SNase cell line had a very affirmative anti viral efficacy.

4. Discussion

JE is a serious disease prevalent throughout Asia and is transmitted to humans by mosquito bite (Solomon, 2003). Pigs and wild boars are important amplifier hosts for the virus (Nidaira et al., 2007; Nitatpattana et al., 2008). Vaccination of swine, therefore, can help prevent disease in humans. DNA vaccines against JEV have showed great potential as preventative agents for their ability to elicit potent humoral and cytotoxic cellular immune responses against the plasmid-encoded protein in a broad range of hosts (Libraty et al., 2002). But there is still no specific therapy to avoid infected by human and animals. Therefore, it is urgent to develop a new and effective anti viral strategy against JEV.

Natsoulis and Boeke (1991) first proposed the new CTVI anti viral strategy using fusion of viral capsid proteins and nuclease. Since then, this strategy was widely used on a various of viruses. These studies showed that CTVI was specific and efficient and could be developed as anti viral drugs. In addition, CTVI had several advantages over other anti viral strategies, for example: RNA-based inhibitors, like antisense RNA or ribozymes. First, it is highly efficient, because, at least in theory, even one incorporated active nuclease molecule could disable a virion. The nuclease chosen as the effector molecule in CTVI is a protein enzyme, which is catalytically more efficient than a ribozyme. Second, escape mutants rarely arise because the carrier, usually a capsid protein, plays an important role in the viral life cycle. No data have been reported implying the existence of escape mutants during CTVI. Furthermore, CTVI could be used either for recombinant protein drugs or in gene therapies superior to nucleic-acid–based strategies, so that it is a very sidely used anti viral strategies.

In this study, the data showed Cap-SNase fusion proteins incorporated into virions that are released into the extracellular milieu

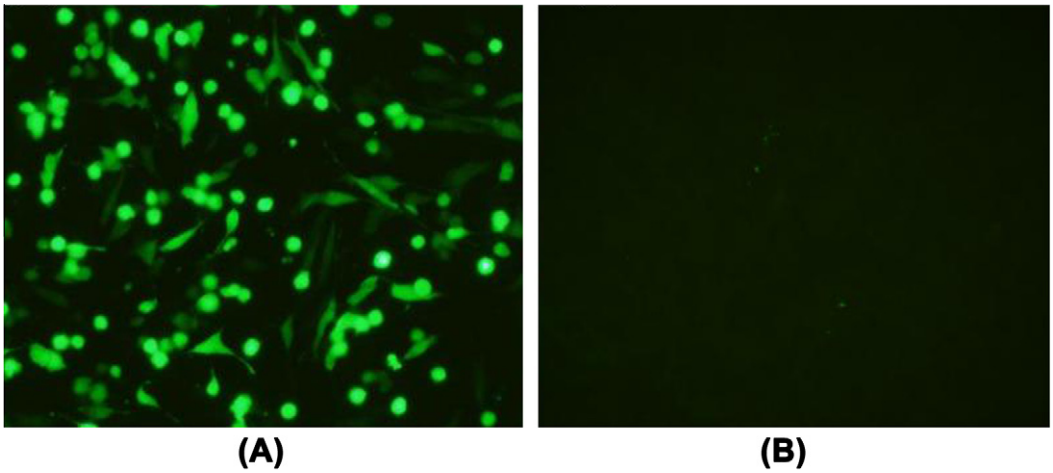

Fig. 4. Indirect immunofluorescence assay (IFA) analysis of BHK-21/Cap-SNase cells (A) and BHK-21 cells (B). Transfected cells beyond passage of 15 generations were fixed in acetone for 30 min at −20 °C and washed with PBS. Rabbit anti-JEV antibody (1:100) was applied as the first antibody and FITC-goat anti rabbit IgG as the second antibody. (A) Cap-SNase fusion proteins were detected by fluorescence signals in the BHK-21/Cap-SNase cells; (B) there were no fluorescence signals in BHK-21 control.

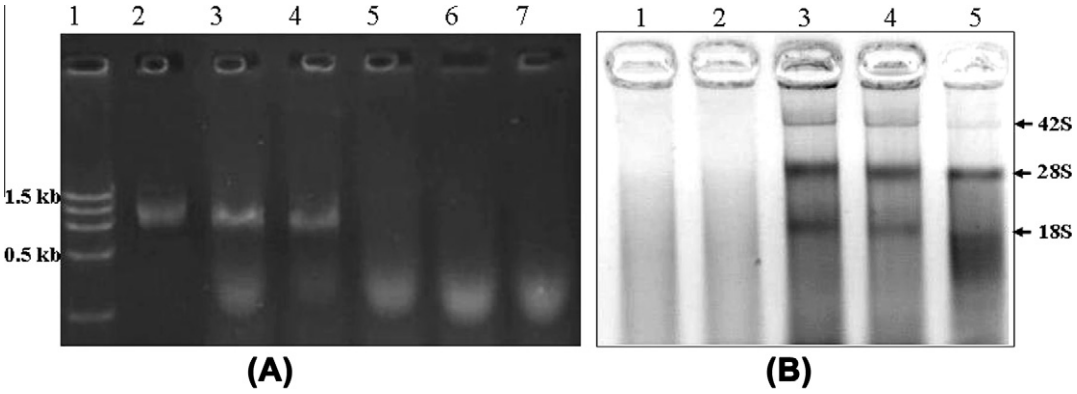

Fig. 5. In vitro nucleic acid digestion assay for analysis of nuclease activity. A. DNA digestion assay for analysis of nuclease activity. With plasmid DNA as substrates, the nuclease activity was determined by the disappearance of the plasmid DNA when incubated with various cell lysates. Lane 1. DNA Marker DL 15000; Lane 2. pcDNA-3.1/V5-His; Lane 3. BHK-21 cell lysates with Ca^{2+}; Lane 4. Standard SNase 0.8 pg without Ca^{2+}; Lane 5. Standard SNase 0.8 pg with Ca^{2+}; Lane 6–7. cell line lysates 5 μl and 7.5 μl with Ca^{2+}, respectively. The results of Lane 6–7 showed that DNA could be digested with Cap-SNase fusion proteins of cell line lysates and the nuclease activity was similar to that of 0.8 pg standard SNase. B. The digestion assay of total RNA from virus-infected cells for analysis of nuclease activity. Lane 1. cell line lysates 7.5 μl with Ca^{2+}; Lane 2. Standard SNase 0.8 pg with Ca^{2+}; Lane 3. Standard SNase 0.8 pg without Ca^{2+}; Lane 4. BHK-21 cell lysates with Ca^{2+}; Lane 5. total RNA from virus-infected cells. The result of Lane 1 showed that virus 42S RNAs and ribosomal RNAs (28S and 18S) could be digested with Cap-SNase fusion proteins of cell line lysates and the nuclease activity was similar to that of 0.8 pg standard SNase.

should become enzymatically active as a result of high Ca^{2+} concentrations and thus should degrade encapsidated viral RNA. The results of Q-PCR and plaque assay, western blot analysis all showed that Cap-SNase fusion proteins had a expected anti viral efficacy both in JEV mRNA expression and viral load in vitro. It was hypothesized that the JEV Cap-SNase proteins would be incorporated within the nascent virions together with native JEV Cap proteins during virion assembly. The internal localization of SNase was based on analysis of the progeny virions. The nuclease activity under denaturing conditions strongly supported the fact that SNase was located on the interior of the virions. However, the mechanism of calcium entry into the virion is unknown but is unlikely to be via a specific uptake system.

In conclusion, BHK-21/Cap-SNase cell line with stable expression of Cap-SNase fusion proteins was estabilshed to inhibit JEV spread. The protein-based CTVI strategy we used had several advantages over other anti viral strategies involving RNA-based inhibitors, like antisense RNA or ribozymes. The application of this anti viral strategy to JEV-infected cells is an important initial goal of this project. Otherwise the more complex project would be to use CTVI to treat JEV infection in humans and swines. Nowadays,

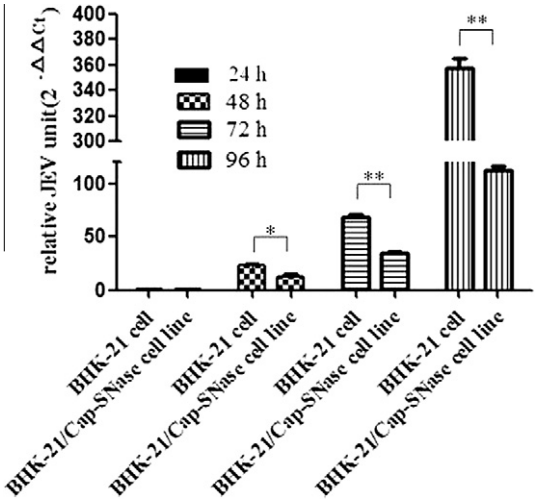

Fig. 6. Inhibition of JEV mRNA expression in BHK-21/Cap-SNase cell line was determinded by real-time RT-PCR. At 48 hpi, the inhibition of virus proliferation in the BHK-21/Cap-SNase cell was considered significant ($P < 0.05$). At 72 and 96 hpi, this inhibition of virus proliferation were oberversed more significant ($P < 0.01$).

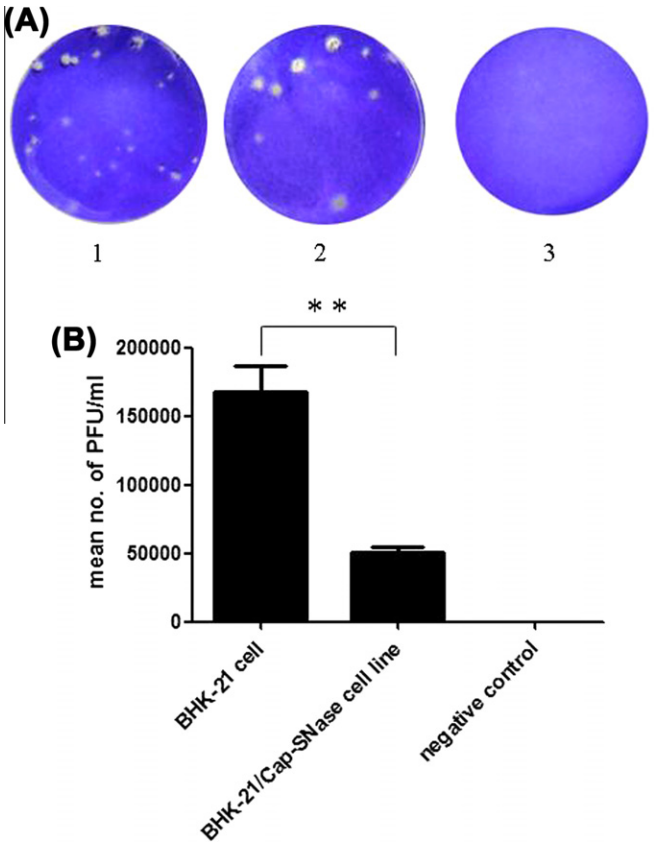

Fig. 7. The results of plaque reduction assay. (A) Plaque assay to subject the effect on reduction of viral load in vivo due to BHK-21/Cap-SNase cell line. (1). BHK-21 cell line infect JEV NJ08 stain at 96 hpi; (2). BHK-21/Cap-SNase cell line infect JEV NJ08 stain at 96 hpi; (3). negative control. (B) the BHK-21/Cap-SNase cell line reduces the viral load in vivo. Significant reduction in viral titer was observed following BHK-21/Cap-SNase cell line, as compared to JEV-infected BHK-21 cells ($P < 0.01$). The error bars represent the SEMs from four independent experiments ($n = 4$).

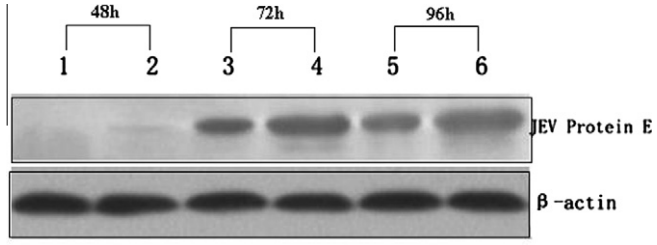

Fig. 8. JEV E protein in BHK-21 cells and BHK-21/Cap-SNase cells were determined to investigate the anti viral effect by Western blot analysis. Lanes 1,3,5 showed the JEV E protein in BHK-21/Cap-SNase cells after 48, 72 and 96 hpi, respectively, but Lanes 2,4,6 showed the JEV E protein in BHK-21 cells after 48, 72 and 96 hpi, respectively. The difference of E protein bands in Lanes 1 and 2, Lands 3 and 4, Lands 5 and 6 could be relatively distinguish, respectively.

we are planning to operate an animal model in vivo for evaluating this anti viral therapy.

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