Journal of Virological Methods 209 (2014) 82–85

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

Production of polyclonal antibody to a recombinant non-structural protein Nsp1a of human astrovirus

Chang Liu[a], Wen-Hui Liu[b], Li-li Kan[c], Xin Li[b], Yong-gang Li[b], Wei Zhao[b,*]

[a] 1st affiliated hospital endocrinology, Liaoning Medical University, Jinzhou, 121000 Liaoning, China
[b] Liaoning Medical University, Jinzhou, 121200 Liaoning, China
[c] Panjin Liaohe Oilfield General Hospital, Panjin, 124010 Liaoning, China

Article history:
Received 20 May 2014
Received in revised form 2 September 2014
Accepted 9 September 2014
Available online 17 September 2014

Keywords:
Human astrovirus
Non-structural protein
Polyclonal antibodies
Escherichia coli expression
Recombinant nsP1a protein

A B S T R A C T

Human astrovirus (HAstV) are important pathogens that cause acute viral diarrhea in infants. Little is known about the mechanisms of astrovirus-induced diarrhea. Previous studies have suggested that an apoptosis inducer may be encoded in the non-structural protein (nsP1a) of astrovirus and contribute to virus-induced diarrhea. To study the biological function of nsP1a and to gain further insight into nsP1a protein-host cell interactions, good quality antibodies must be produced. The nsP1agene of HAstV-1 was cloned into a bacterial expression vector Pgex-6P-1. The recombinant plasmid Pgex-6P-nsP1a was transformed into *Escherichia coli* BL21 (DE3) and expressed as a fusion protein that contains N-terminal GST tags. The expressed recombinant protein was purified and used as an antigen to produce an nsP1a antiserum in rabbits. ELISA was used to detect the titer of specific antibodies. Specificity activity was detected by Western blot and immunofluorescence analysis. The titer of specific antibodies was up to 1:30,000. Western blotting and immunofluorescence analysis indicated that the polyclonal antibody could recognize specifically the HAstV-1 nsP1a protein.

Human astrovirus (HAstV) are causative agents of viral gastroenteritis mainly in children (Kirkwood et al., 2005; De Benedictis et al., 2011). To date, eight serotypes, namely, HAstV-1 to HAstV-8, have been identified. However, several divergent new astroviruses (MLB1/MLB2, VA1/VA2/VA3, and HMO A/B/C) were recently discovered in stool samples from patients with diarrhea (Finkbeiner et al., 2008a,b, 2009a,b,c; Kapoor et al., 2009) and without diarrhea (Walter et al., 2001; Wolfaardt et al., 2011). HAstV is a small, single-stranded, positive-sense RNA virus. The genome is comprised of three open reading frames (ORFs): ORF1a, ORF1b, and ORF2. ORF1a, and ORF1b encode the nonstructural protein (Nsps), whereas ORF2 encodes the structural protein (capsid protein) (Matsui et al., 2001).

The functions of nonstructural protein encoded in ORF1a (nsP1a) of astrovirus have been suggested to be involved in many different functions, including apoptosis induction (Guix et al., 2004a), activate caspases necessary for capsid maturation (Mendez et al., 2004; Banos-Lara Mdel and Mendez, 2010), and RNA replication regulation (Guix et al., 2005). In the nsP1a C-terminal end, called the nsP1a/4 protein, several function domains have been described, such as coiled-coil regions, death domain, a nuclear localization signal, and a hypervariable region (Guix et al., 2004b). The function of the nsP1a protein in viral replication and interaction with the viral RNA or host cell were very important for researching the mechanisms of HAstV induced cell injury. However, these were not commercial antibody target nsP1a proteins. In this study, good quality anti-nsP1a antibodies that may be applied in biological functions and provide insight into nsP1a protein-host cell interactions studies were prepared.

The HAstV-1 China isolate JZ strain (GenBank, NCBI accession KF211475) was used to design the primers 1a-F: 5-CGCGGATCCATGGCACACGGTGAGCCATACTATAGT-3 and 1a-R: 5-CCGCTCGAGATGAGTGGTAGGTTTGGGCCCCTTGG-3. The primers are amplifications of the full-length nsP1a protein gene (2781 bp) fused with restriction sites (*BamH*I, forward and *Xho*I, reverse) for directional cloning to the Pgex-6P-1 vector (GE Healthcare Bio-Sciences, Little Chalfont, UK). The stool of the HAstV-1 JZ strain was used as a template for RNA isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions; cDNA synthesis was performed using the Primescript™ first reverse transcriptase (TakaraBio, Otsu, Japan) according to the protocol established by the manufacturer. The cDNA template (10 μL) was used in a 50 μL reaction mixture and amplified using MMLV taq (TakaraBio, Otsu, Japan). The amplification program involved an

* Corresponding author at: Liaoning Medical University, No. 40, the Third Section of Songpo Rd., Jinzhou City, 121200 Liaoning Province, China.
E-mail address: zhaowei8072@163.com (W. Zhao).

http://dx.doi.org/10.1016/j.jviromet.2014.09.007
0166-0934/

initial 5 min step at 94 °C, followed by 40 cycles of 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 3 min. A final elongation step at 72 °C for 5 min was performed. The PCR products were detected on an agarose gel containing 0.5 μg/mL ethidium bromide, and amplicons of the nsP1a gene were excised and extracted using the AxyPrep DNA gel extraction kit (Axygen, CA, USA). The purified PCR product (2781 bp) was then cloned into *Xho*I and *Bam*H I sites of Pgex-6P-1 to generate the plasmid Pgex-6P-nsP1a. The recombinant plasmid Pgex-6P-nsP1a was transformed into competent *E. coli* BL21 (DE3) and grown overnight in 5 mL of Luria-Bertani broth supplemented until the optical density (OD600) reached 0.6–0.8. The expression of Pgex-6P-nsP1a was induced with 0.1–1 Mm of isopropyl-β-D-thiogalactopyranoside (IPTG) at 25–37 °C on a shaker at 220 rpm. It was then analyzed via polyacrylamide gelelectrophoresis (SDS-PAGE). After the expression of Pgex-6P-nsP1a fusion protein was confirmed, the supernatant containing fusion Pgex-6P-nsP1a protein was purified using Glutahione Sepharose 4B (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the manufacturer's instructions.

The antisera against the expressed nsP1a were prepared in three New Zealand rabbits, and intramuscular injections of 2 mg of purified protein were used. The protein (1 mg) was emulsified with an equal volume of Freund's complete adjuvant for the first injection and with Freund's incomplete adjuvant for subsequent injections (500 μg of the purified protein). For the final injection, the antigen (500 μg) was diluted in PBS. The rabbits were bled two weeks after the final injection. The serum fractions were collected and stored at −20 °C. The titer of specific nsP1a polyclonal antibody was detected via indirect ELISA (I-ELISA). The specificity activity was detected further via Western blot. To determine whether polyclonal antibodies can used to detect the expression of nsP1a protein, 293 T cells were seeded onto a 6-well plate and grown to confluence until it reached approximately 90%. The cells were transfected with 4 μg of pcDNA3.1 (+)-nsP1a and pEGFP-N3-nsP1a (control plasmid) by using lipofectamin 2000 transfected reagent (Invitrogen, Carlsbad, CA) according to instructions. Immunofluorescence analysis was then performed. The pcDNA3.1 (+)-nsP1a and pEGFP-N3-nsP1a plasmid were constructed in another research (data not shown). Simultaneously, the 293 T cells were infected with human astrovirus 3 strain (provided by Doctor Man-qing Liu from the Divisions of Virology, and Wuhan Centers for Disease Prevention and Control, China) at a multiplicity of infection (moi) of 5 according to previous reports. After 24 h of infection, 4% paraformaldehyde was added to the monolayers. All the cells were detected via the immunofluorescence technique by using the nsP1a polyclonal antibody prepared in this study. FITC-conjugated goat anti-rabbit antibody and Alexa Fluor 594-conjugated goat anti-rabbit antibody were used as secondary antibodies.

The RT-PCR of nsP1a protein gene with specific primers generated a 2781 bp fragment, which was cloned into Pgex-6P-1 vector. The resulting plasmid, Pgex-6P-nsP1a, was sequenced. Analysis showed that the nsP1a gene was inserted in a frame with intake N-end GST tags. After IPTG induction, competent *E. coli* BL21(DE3) that harbor Pgex-6P-nsP1a efficiently produced recombinant fusion proteins that were absent from un-induced cultures (Fig. 1 lane 1 and lane 6–7). The GST derived from the vector fused to the N-terminal of expressed protein. Thus, recombinant viral nsP1a should have a higher molecular weight than wild nsP1a (103 kDa). The recombinant nsP1a-GST fusion protein had a relative molecular weight (MW) of 129 kDa, including 26 kDa GST protein. SDS-PAGE analysis of soluble fraction and cell debris pellet from IPTG-induced bacterial cultures showed that the major part of nsP1a fusion protein was present in the soluble fraction; the maximum level of 1 mM for the IPTG-induced cultures were observed after 16 h of induction at 30 °C (Fig. 1, lines 2–8). The recombinant nsP1a protein was purified using Glutahione Sepharose 4B. SDS-PAGE

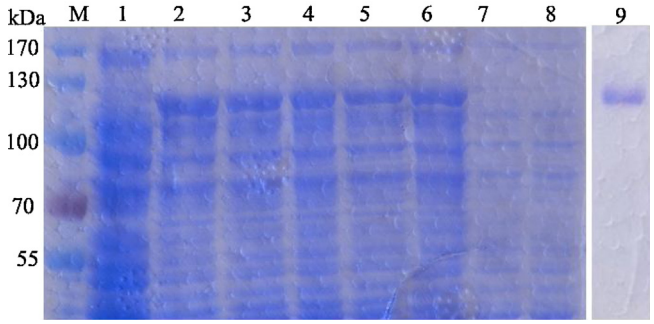

Fig. 1. Electrophoretic analysis of expressed human astrovirus nonstructural protein nsP1a in *Escherichia coli*. The BL21 (DE3) *Escherichia coli* cells that harbor Pgex-6P-nsP1a were induced using 0.1 mM–1 mM IPTG for 16 h at 30 °C at 220 rpm. The proteins were analyzed using 10% Tricine SDS-PAGE, and the gels were stained with coomassie brilliantblue G-250. Lane 1: Total protein from uninduced cells; lanes 2–6: soluble cell protein fraction, IPTG concentrate was 0.1, 0.3, 0.5, 0.7, and 1 mM, respectively. Lanes 7–8: Total protein from induced cells of insoluble protein fraction; Lane 9: Purified recombinant Pgex-6P-nsP1a protein. M: protein standard marker (Thermo Scientific, Waltham, MA, USA).

analysis of purified protein revealed the presence of one distinct band of 129 kDa after staining, as expected (Fig. 1, line 9), which consists of 26 kDa of GST tags. The yield of nsP1a fusion protein purified from 300 mL of *E. coli* cells was about 3.0 mg after estimation via Bradford assay. The anti-nsP1a sera had titers of 1:30,000 when tested in an indirect ELISA with purified protein. The antisera strongly reacted with a homologous recombinant antigen. The polyclonal antibodies reacted with the homologous recombinant nsP1a, and Western blot revealed a strong band at a position that corresponds to 129 KDa, which is the expected molar mass of nsP1a (Fig. 2). To determine whether polyclonal antibodies can used to detect the expression of nsP1a proteins and virus infected cells, immunofluorescence analysis was performed. After 48 h of transfection, nsP1a polyclonal antibody (diluted 1:3000) can be used to detect the expression of nsP1a protein in vitro. Green fluorescence was noticeable in the transfected cells (Fig. 3A and B). After 24 h of HAstV-3 infected cells, nsP1a protein was also detected and red fluorescence was noticeable in the infected cells (Fig. 3C and D).

Human astrovirus non-structural protein encoded nsP1a function remained elusive. Computational analysis of HAstV nsP1a suggested the existence of many posttranslational modification motifs especially the C-terminal of nsP1a (Fuentes et al., 2011). A transient expression system was used to reveal the number of transfected cells that express cells that confirm the presence of a proapoptotic protein in nsP1a (Finkbeiner et al., 2009a,b,c). To study the biological function of nsP1a or screen the interaction protein of nsP1a and host cell proteins, a full-length nsP1a protein

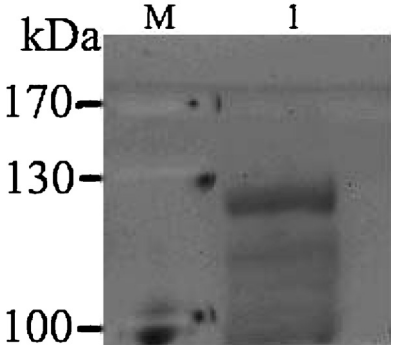

Fig. 2. Western blots analysis of nsP1a protein using recombinant anti-nsP1a antibodies (lane 1). M: protein standard marker (Thermo Scientific, Waltham, MA, USA).