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Identification of adenovirus neutralizing antigens using capsid chimeric viruses

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ABSTRACT

Human adenoviruses (HAdV) 3 and 7 can cause acute respiratory disease epidemics and outbreaks. Identification of neutralizing epitopes is vital for surveillance and vaccine development. In this study, we generated the recombinant capsid-chimeric human adenoviruses rAd3E-Fk7, containing the Ad3E backbone and the HAdV-7 fiber knob, and rAd3E-H7Fk7, which contain an Ad3E backbone but HAdV-7 hexon and fiber knob. *In vitro* neutralization tests with these chimeric adenoviruses using both mouse and human antisera indicated that hexon and fiber knob are the major targets recognized by neutralizing antibodies against HAdV-3 or HAdV-7, and other capsid proteins including the penton base and fiber shaft may not contribute to neutralizing antibody responses. In conclusion, both hexon and fiber knob structures in HAdV-3 and HAdV-7 may be the proteins which induce neutralizing antibody responses and thus may be important for adenovirus vaccine and drug development.

1. Introduction

Human adenoviruses (HAdV), of the family *Adenoviridae*, genus *Mastadenovirus*, are non-enveloped double-stranded DNA viruses that cause acute respiratory disease (ARD), gastroenteritis, pneumonia, and keratoconjunctivitis (Chuang et al., 2003; Yu et al., 2013b; Zou et al., 2012). There are 51 serotypes recognized to date(Lenaerts et al., 2008), and at least 83 genotypes of human adenoviruses defined using a new paradigm based on genomics, which are divided into seven human adenovirus species, HAdV-A–HAdV-G, (species Human mastadenovirus A through G) (Espinola et al., 2017; Jones et al., 2007; Seto et al., 2011). The B1 adenoviruses HAdV-3 and HAdV-7 (species *Human Mastadenovirus B*, serotypes 3 and 7), which cause ARD, have caused outbreaks around the world(Choi et al., 2005; James et al., 2007; Lai et al., 2013; Mitchell et al., 2000; Selvaraju et al., 2011; Zhang et al., 2006). Globally, HAdV-3 is among the most common types implicated in HAdV infections in children and adults. Epidemic HAdV-7 infections have been reported in the United States, Canada, Latin America, Australia, Israel, Korea, Japan, China, the Philippines and globally (Lynch and Kajon, 2016).

The adenoviral capsid is composed of three major proteins: hexon, fiber, and penton base. Gall et al (1996) reported that antibodies against HAdV-7 fiber were not sufficient to neutralize chimeric HAdV-5/F7 *in vivo* with HAdV-B7 fiber gene replacing the HAdV-5 fiber gene (Gall et al., 1996). The hexon protein carries the major type-specific antigenic determinants recognized by neutralizing antibodies (NAbs) (Bradley et al., 2012; Su et al., 2016; Sumida et al., 2005; Tian et al., 2011; Yu et al., 2013a). Sumida has reported that HAdV-5-specific NAbs are directed primarily against the HAdV-5 hexon protein in both humans and mice (Sumida et al., 2005). Bradley described a chimeric HAdV-5, in which both the hexon hypervariable regions (HVRs) and the fiber knob were exchanged, that almost completely evaded neutralization by HAdV-5-specific NAbs, both *in vitro* and *in vivo* (Bradley et al., 2012). These studies were using HAdV-5 of species C. Our lab also found that HAdV-3-specific NAbs are directed primarily against the hexon protein (Su et al., 2016; Tian et al., 2011; Zhao et al., 2015). In our previous research, the chimeric adenoviral vector, HAdV-3/H7, was constructed by replacing the HAdV-3 hexon gene (H3) with the hexon gene of HAdV-7 (H7) (Tian et al., 2011). We found that HAdV-3- and HAdV-7-specific NAbs were directed primarily against the hexon proteins both *in vitro* and *in vivo*. However, it remains unclear whether the fiber or penton base proteins of the HAdV-3 and HAdV-7 viruses induce neutralizing antibodies.

In the current study, we successfully constructed two recombinant

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adenoviruses, named rAd3E-Fk7, by replacing the HAdV-3 fiber knob (Fk3) with the corresponding region from HAdV-7 (Fk7), and rAd3E-H7Fk7, by replacing the rAd3E-Fk7 hexon gene with H7. We also investigated the functional significance of hexon and fiber knob NAbs in human serum samples and in mice sera. This research may be helpful for the development of adenovirus vaccines and neutralizing monoclonal antibody.

2. Materials and methods

2.1. Viruses and cells

The plasmids and viruses used in this study were maintained in our laboratory, provided by the State Key Laboratory of Respiratory Disease, Guangzhou, China. Adenoviruses were cultured in A549 cells, which were purchased from the ATCC and subsequently maintained in our lab. A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) with Penicillin-G 100 UmL-1, streptomycin 100 μg mL-1 and 10% (v/v) fetal bovine serum (FBS) (Invitrogen).

Adenoviruses were purified by standard CsCl gradient centrifugation. HAdV-3 GZ-1(GenBank no. DQ099432), a clinical isolate in our lab, was cultured in A549 cells. HAdV-7-CQ1198 (GenBank no.JX625134.1) was provided by the Children's Hospital, Chongqing Medical University (Chongqing, China). *Escherichia coli* BJ5183 and Top 10 cells were purchased from Takara (Dalian, China).

2.2. Human serum samples

A total of seven serum samples from healthy individuals were collected at random in 2015 by the First Affiliated Hospital of Guangzhou Medical University in Guangzhou, Southern China. Donors were aged from 20 to 49 years with a sex ratio of 1:1, without any other participant identifiers. The data were analyzed anonymously.

2.3. Construction of recombinant plasmids

A recombinant adenovirus, rAd3E, encoding the HAdV-3 GZ01 genome (GenBank accession no. DQ099432) and an enhanced green fluorescent protein (EGFP) were generated (Fig. 1A) as previously described (Zhang et al., 2009).

The recombinant adenovirus, pAd3E-Fk7, with Fk3 being replaced with Fk7, was constructed (Fig. 1I).

Firstly, the shuttle vector, pshuttle-A3-fiber, for the HAdV-3 fiber replacement was constructed as follow: polymerase chain reaction (PCR) was performed using the primers Fiber-L-Rsr II: (5′-CCATCGAT CGGTCCGCAGCGACAAAAAT-3′), Fiber-R-Pme I: (5′-CCATCGATGTTT AAACCACACCTCATT-3′) (Fig. 1A,1B). The PCR product was digested with Cla I and then ligated as pShuttle-A3-fiber (Fig. 1C).

Then, the Fk7 gene fragment was obtained by PCR from HAdV-7 genome using the primers Fk7-F: (5′-CTCAAAAATA ACACTCTATGGA CAGGAGTTAACCCCAC-3′), Fk7-R: (5′-GTCATCTTCTGTAATGTAGTAA AAGGTAAATGGAGAGGTG-3′) (Fig. 1E). The fragment ps-A3-fiber was obtained by PCR using pshuttle-A3-fiber as template with the primers C-Fiber-F (5′-ATTACAGAAGATGACTGACAACA-3′) and C-Fiber-R (5′-AGTGTTATTTTTGAGTGCAATAGAAT-3′) (Fig. 1D). The resulting fragment Fk7 and fragment ps-A3-fiber which have 15bp homologous arms were then recombined with 1:1 DNA quantity ratio to get pshuttle-A7Fk with Exnase according to the ClonExpress Entry One Step Cloning Kit (Vazyme, Nanjing, China) (Fig. 1F), and subsequently co-transfected into Top 10 cells.

Thirdly, the fragment ps-A7Fk was obtained by PCR using pShuttle-A7Fk as template with the primers fiber knob-F (5′-ACTAGTATACGA GCTCGGTCCGCAGCGACAAAAAT-3′) and fiber knob-R (5′-GTCATCTT CTGTAATGTAGTAAAAGGTAAATGGAGAGGTG-3′) (Fig. 1G). The pBRAd3E plasmid encoding the Ad-3 GZ-01 genome and EGFP with an E3 region deletion was constructed as previously described (Zhang et al., 2009). The fragment pBRAd3E-R/P was obtained as following: pBRAd3E was digested with both RSr II and Pme I, and the larger fragment was purified by gel extraction kit (Takara, Dalian, China) (Fig. 1H). The fragment ps-A7Fk by PCR and the fragment pBRAd3E-R/P, which have 15bp homologous arms were then recombined to get plasmid pAd3E-Fk7 with the same DNA quantity using Exnase according to the ClonExpress Entry One Step Cloning Kit (Vazyme, Nanjing, China) (Fig. 1I), and subsequently co-transfected into Top 10 cells.

The shuttle vector, pBRLH7S, for the H7 replacement was constructed as previously described (Tian et al., 2011). H7 fragment was obtained by PCR from pBRLH7S with the primers A3-Avrllr (5′-TGCC TAGGAGAACACAATGGCCAG-3′) and A3-Paclu (5′-GCCGTCGCTGCTA TTAATTAAATATGGA-3′) (Fig. 1K). The pAd3E-Fk7 plasmid was digested with AvR II and Pac I (Fig. 1G), and the fragment pAd3E-Fk7-AvR II- Pac I was purified and recombined with the fragment H7 using Exnase according to the ClonExpress Entry One Step Cloning Kit (Vazyme, Nanjing, China) (Fig. 1L). The resultant clones pAd3E-H7Fk7 and pAd3E-Fk7 containing Fk7 were designated and selected by PCR using primers GFP-F(5′-ATGGTGAGCAAGGGCGAGGAG-3′) and AdV7-fk-R(5′-GTCATCTTCTGTAATGTAGTAAAAGGTAAATGGAGAGGTG-3′) (Fig. 2A).The resultant clones pAd3E-H7Fk7 containing H7 were designated and selected by PCR using primers a7u (5′-ACAGCAGGAGAA GAAAGAG-3′) and A7r2 (5′-CTTTTTCCCATCCATTGTCTTTAGAT-3′) (Fig. 2 B). Constructs were confirmed by restriction digestion and sequencing analysis.

2.4. Virus rescue and preparation

To rescue modified rAd3E-Fk7 and rAd3E-H7Fk7 viruses, pAd3E-Fk7 and pAd3E-H7Fk7 were digested with AsiS I (NEB, USA). Linearized genomic DNA was transfected into A549 cells grown in 24-well plates, using lipofectamine 3000 (Invitrogen). The transfected cells were cultured at 37 °C, 5% CO_2 for 6 days. Cells were then subjected to freeze/thaw cycles and the lysate was used to infect fresh cultures of A549 cells. To prepare the viral stocks, a total of twenty culture dishes (100 mm) of A549 cells for each recombinant virus were infected with rAd3E, rAd3E-FK7 or rAd3E-H7Fk7, and harvested at 96 h post-infection. Viruses were purified by standard CsCl gradient centrifugation. The virus particle (VP) titers were determined by spectrophotometry using a conversion factor of 1.1×10^{12} VP per absorbance unit at 260 nm. Recombinant virus stocks were stored in virus storage buffer (10 mM Tris pH 8.0, 2 m M Mg Cl2, 4% sucrose, 10% glycerol) at −80 °C. The wild-type viruses HAdV-3 and HAdV-7 were also cultured in A549 cells, purified and stored at −80 °C. The tissue culture infectious doses ($TCID_{50}$) of the viruses were determined following the Reed and Muench method(Reed, 1938).

2.5. Growth characteristics of recombinant viruses

To evaluate the replication characteristics of virus, A549 cells were infected with 1×10^9 genome copies of purified virus HAdV-3, rAd3E-Fk7 and rAd3E-H7Fk7, and washed with PBS and then harvested at 12hr-, 24hr-, 36hr-, 48hr-, 72hr-, and 96hr-post-infection. The number of viral genome copies at each time point was determined by real-time quantitative PCR (Q-PCR) using a universal adenovirus Q-PCR kit (Hexin Corporation, Guangzhou, China) on an Applied Biosystems 7500 real-time PCR system. Each assay was performed three times in duplicate.

2.6. Western blotting

Recombinant HAdV-7 fiber knob (Fk7; amino acids 123–325 of the adenovirus genome) and Fk3 peptide (amino acids 124–319) were expressed in *E. coli* and purified by affinity chromatography using Ni-NTA