Antiviral Research 121 (2015) 145–151

Contents lists available at ScienceDirect

Antiviral Research

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

Recombinant influenza virus carrying human adenovirus epitopes elicits protective immunity in mice

Penghui Yang [a,b,1], Tieling Li [c,1], Na Liu [a,1], Hongjing Gu [b], Lina Han [c], Peirui Zhang [a], Zhiwei Li [a], Zhaohai Wang [a], Shaogeng Zhang [a,*], Xiliang Wang [b,*]

[a] Beijing 302 Hospital, Beijing 100039, China
[b] Beijing Institute of Microbiology and Epidemiology, State Key Laboratory of Pathogen and Biosecurity, Beijing 100071, China
[c] Chinese PLA General Hospital, 1000853, China

ARTICLE INFO

Article history:
Received 4 June 2015
Revised 18 June 2015
Accepted 22 June 2015
Available online 23 June 2015

Keywords:
Influenza virus
HAdV
Hexon protein epitopes
Viral vector

ABSTRACT

Human adenoviruses (HAdVs) are known to cause a broad spectrum of diseases in pediatric and adult patients. As this time, there is no specific therapy for HAdV infection. This study used reverse genetics (RG) to successfully rescue a recombinant influenza virus, termed rFLU/HAdV, with the HAdV hexon protein antigenic epitope sequence inserted in the influenza non-structural (NS1) protein gene. rFLU/HAdV morphological characteristics were observed using electron microscopy. Furthermore, BALB/c mice immunized twice intranasally (i.n.) with 10^4 TCID50 or 10^5 TCID50 rFLU/HAdV showed robust humoral, mucosal, and cell-mediated immune responses *in vivo*. More importantly, these specific immune responses could protect against subsequent wild-type HAdV-3 (BJ809) or HAdV-7 (BJ1026) challenge, showing a significant reduction in viral load and a noticeable alleviation of histopathological changes in the challenged mouse lung in a dose-dependent manner. These findings highlighted that recombinant rFLU/HAdV warrants further investigation as a promising HAdV candidate vaccine and underscored that the immuno-protection should be confirmed in primate models.

1. Introduction

Adenovirus, first isolated in the 1950s, is a leading cause of acute respiratory disease (ARD) and is associated with hospitalizations and mortality for pneumonia. These outcomes have been attributed to many of the 55 known human adenoviral (Ad) serotypes distributed among six subgroups (A–F), but they most commonly involve viruses of subgroups 3 and 7 in China (Xue et al., 2014). At this time, there is no specific therapy for adenoviruses. The first generation formalin-inactivated adenoviral vaccine was prepared in 1956 (Li et al., 2009). However, many shortcomings, including lot-to-lot variations, contamination with simian virus 40, and the potential oncogenicity of Ad3 and Ad7, resulted in their failure in 1963. Later, Ad4 and Ad7 vaccines made from human diploid cells were in continuous use and reduced the incidence of acute respiratory illness in US military recruit populations. In 2001, the U.S. Department of Defense (DoD) signed a contract with BarrLaboratories Inc. to resume production of HAdV vaccines, which were oral, live, and enteric-coated vaccine tablets against adenovirus serotypes 4 and 7 (Blasiole et al., 2004). The licensed live human adenovirus oral vaccine was approved by the U.S. Food and Drug Administration in March 2011 for U.S. military personnel only. Due to potential safety risks, the vaccine is not available to the public.

Structurally, AdV is non-enveloped, regular, and icosahedral. The virion has 20 triangular faces and 12 vertices, with 240 hexons and 12 pentons (Gregory et al., 2011). The hexon protein of an adenovirus is considered the major neutralizing protective antigen, and it is an important coat protein in the induction of immune responses *in vitro* and *in vivo* (Sumida et al., 2004, 2005). This study is the first investigation of the construction and characterization of a recombinant influenza virus carrying the hexon protein epitopes of HAdV, termed rFLU/HAdV. Using reverse genetics, a recombinant influenza virus was rescued using influenza virus PR8 as vector, with hexon protein epitopes of HAdV inserted into the position of non-structural protein NS1. The immune response and protective immunity against wt HAdV were determined in BALB/c mice immunized with rFLU/HAdV.

* Corresponding authors.
E-mail addresses: zhangsg302@hotmail.com (S. Zhang), xiliangw@126.com (X. Wang).
[1] These authors contributed equally to this paper.

http://dx.doi.org/10.1016/j.antiviral.2015.06.015
0166-3542/

2. Materials and methods

2.1. Viruses and cells

Wild-type (WT) HADV HAdV-3 strain (Strain BJ809, GenBank No. AY854809.1) and HAdV-7 strain (Strain BJ1026, GenBank No. AF621026.1) were obtained from Beijing Children Hospital and cultured in human laryngeal epithelial (HEp-2) cells (ATCC, Manassas, Virginia, USA). HEp-2, MDCK, A549, and CNE-2Z cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). WT influenza virus strain A/PR/8/34 (PR8) was inoculated and propagated in the allantoic cavity of 9–11-day-old SPF chicken embryos (Beijing Laboratory Animal Center). Allantoic fluids were harvested after 3 days and stored at −80 °C until use.

2.2. Construction of recombinant plasmid NS1-hexon

HAdV-3 and HAdV-7 hexon protein epitopes (Loop1/115-310: IVTAGEERAVTT GKDITADNKP, ESWTDTDGTNEKFG, NRKVKPTTEGGVETE, GREAADAFSPE; Loop2/400-510: LDGIGPAK, IKSKDNGWEKDDNVSKS, NVSKSN) were inserted into the NS1 gene of PR8 influenza virus (Tian et al., 2013; Xue et al., 2014). Notably, there are six GPG rigid linkers between the eight different epitopes. Briefly, an overlapping TAATG stop–start code cassette was introduced into the NS gene. The coding sequences of HAdV hexon epitopes were blunt end cloned downstream of the stop–start cassette. These sequences were synthesized by Beijing GENEWIZ Company. The cDNA of recombinant plasmid NS1-hexon expressed in vector pHW2000 was cloned by *Bsm*BI enzyme digestion, ligation, and transformation; the positive clones were confirmed by DNA sequencing. The positive eight plasmids carrying the PB2, PB1, PA, HA, NP, NA, M, and NS gene segments of the A/PR/8/34 virus (pHW191–pHW198) were confirmed as described previously (Duan et al., 2014).

2.3. Rescue of recombinant virus rFLU/HAdV from cloned cDNA

The recombinant influenza virus rFLU/HAdV was rescued carrying the NS1-hexon recombinant gene along with the remaining gene segments from the A/PR/8/34 influenza virus (pHW191, pHW192, pHW193, pHW194, pHW195, pHW196, and pHW197) as described by Yang et al. (2011). When a hemagglutination-positive result was detected, recombinant rFLU/HAdV virus supernatant and cell lysates were collected. Viruses were propagated in chicken embryos, concentrated by ultra-filtration (PALL, USA), and purified on a 20–30–60% sucrose gradient. Infective titers were determined and expressed as the 50% tissue culture infective dose in MDCK cells ($TCID_{50}$/ml) using the method of Reed and Muench (Neumann and Kawaoka, 2001).

2.4. Electron microscopy

Recombinant rFLU/HAdV viruses were characterized by electron microscopy. For morphology and size determinations, negative staining of rFLU/HAdV was conducted, followed by visualization by transmission electron microscopy.

2.5. Virus growth

To further examine viral growth curves *in vitro*, MDCK, A549, and CNE-2Z cells were infected with rFLU/HAdV or wild-type PR8 viruses at an MOI of 0.02, respectively. The plates were incubated at 35 °C in 5% CO_2. Cell culture supernatants were harvested every 12 h until 7 h post-infection, and virus titers were assayed and expressed as PFU/ml.

2.6. Quantitative real-time PCR

RNA was extracted from the lungs of rFLU/HAdV-immunized mice at different times post-infection using the RNeasy Mini Kit (Takara). Real-time RT-PCR was performed on an ABI 7500 system (Applied Biosystems) with primers and TaqMan one-step RT-PCR master mix (Applied Biosystems). Viral genomic DNA copy numbers were determined, and samples were normalized to GAPDH as a control. Relative amounts of mRNA were calculated using the comparative C_T method.

2.7. Immunization and challenge in BALB/c mice

Female BALB/C mice (6–8 weeks) purchased from the Animal Experimental Center of AMMS were used in this study. Groups of mice were immunized intranasally twice with either 10^4 or 10^5 $TCID_{50}$ of rFLU/HAdV at 4-week intervals. Blood samples were collected before immunization and on days 28 and 42 after priming. Mice were sacrificed 2 weeks after the second immunization. Meanwhile, nasal and lung lavages were collected for measurement of mucosal sIgA titer. Tissue samples were stored at −20 °C until use. For viral challenge, vaccinated mice were isoflurane-anesthetized and infected i.n. with live 10^9 VPs BJ809 or BJ1026 at 2 weeks after boost, respectively. Mice were observed daily and changes in body weight were recorded. All animal experiments were conducted under the guidelines of the Beijing Institute of Microbiology and Epidemiology Animal Care and Use Committee.

2.8. Neutralization assays

To detect recombinant influenza virus rFLU/HAdV-specific neutralizing antibodies, a hemagglutination inhibition (HI) assay was performed using standard protocols. Briefly, four hemagglutination units (HAU) of influenza virus PR8 were added to a v-bottom 96-well plate containing 0.5% turkey erythrocytes (Webster et al., 1991). HI titers were expressed as the reciprocal of the highest serum dilution that completely inhibited agglutination of the chicken erythrocytes by influenza virus PR8.

Neutralization tests (NT) against HAdV-3 strain BJ809 or HAdV-7 strain BJ1026 were performed using a 50% plaque reduction assay. Mice sera were serially diluted and mixed with an equal volume of BJ809 or BJ1026 (100 PFU) and inoculated on a monolayer of HEp-2 cells in 12-well plates. Plaques were counted and NT antibody titers were analyzed as the reciprocal of the serum dilution that showed a 50% reduction in plaque number.

2.9. HAdV protein-specific ELISA

The enzyme-linked immunosorbent assay (ELISA) was performed in a microtiter plate, as described previously (Farnos et al., 2006; Guo et al., 2003). Plates for the detection of HAdV-specific secretory IgA (sIgA) in nasal and lung lavage fluids were coated with 5 μg/ml inactivated BJ809 or BJ1026 virions. Samples were serially diluted 1:2 in PBS containing 1% bovine serum albumin (BSA) (Serva, BRD) and added to the coated plates. Bound antibodies were detected using goat anti-mouse IgA (Sigma, USA) conjugated to horseradish peroxidase (HRP) and diluted 1:20,000. Plates were stained with TMB (Sigma, USA) as a substrate and read at 450 nm. Each sample was repeated in triplicate.