Virus Research 183 (2014) 67–74

Contents lists available at ScienceDirect

Virus Research

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

Construction and characterization of a recombinant human adenovirus type 3 vector containing two foreign neutralizing epitopes in hexon

Chunyan Xue[a,1], Xingui Tian[a,1], Xiao Li[a], Zhichao Zhou[a], Xiaobo Su[b,*], Rong Zhou[a,**]

[a] State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou 510120 , China
[b] Department of Medical Genetics and Cell Biology, School of Basic Science, Guangzhou Medical University, Guangzhou 510120, China

ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form 25 January 2014
Accepted 31 January 2014
Available online 8 February 2014

Keywords:
Ad vector
Hypervariable region
Epitope display
Immune response

ABSTRACT

The "antigen capsid-incorporation" strategy has been developed for adenovirus-based vaccines in the context of several diseases. Exogenous antigenic peptides incorporated into the adenovirus capsid structure can induce a robust and boosted antigen-specific immune response. Recently, we sought to generate a multivalent adenovirus type 3 (Ad3) vaccine vector by incorporating multiple epitopes into the major adenovirus capsid protein, hexon. In the present study, a multivalent recombinant Ad3 vaccine (R1R2A3) was constructed by homologous recombination, displaying two neutralizing epitopes from enterovirus type 71 (EV71) in hexon. The recombinant virus was confirmed by PCR, immunoblotting, and enzyme-linked immunosorbent assay, and injected into mice to analyze the epitope-specific humoral response. No differences were found between the viruses with two epitopes incorporated into the hypervariable regions (HVR1 and HVR2) of hexon and Ad3EGFP, based on thermostability and growth kinetic tests. Both the epitopes are thought to be exposed on the hexon-modified intact virion surface. The repeated administration of the modified adenovirus R1R2A3 to BALB/c mice boosted the humoral immune response against both epitopes. Immunization with recombinant virus R1R2A3 elicited higher IgG titers and higher neutralization titers against EV71 in vitro than immunization with the modified adenovirus with only one epitope incorporated into HVR1. In this study, the recombinant R1R2A3 virus expressing two exogenous neutralizing epitopes in hexon HVR1 and HVR2 induced specific immune responses to both foreign epitopes. Our study contributes to a better understanding of hexon-modified Ad vector as a multiple-epitope delivery vehicle.

1. Introduction

Adenoviral vectors have been widely used for vaccination against cancer and infectious diseases. However, traditional adenoviral vaccines, designed to express antigens that are encoded as transgenes, have yielded suboptimal clinical results, attributed in part to the preexisting immunity of the recipient to adenovirus type 5 (Ad5), arising from natural adenoviral infection or previously administered Ad5 vectors (Nabs; Schagen et al., 2004; Zaiss et al., 2009; Pandey et al., 2012). For this reason, the "antigen capsid-incorporation" strategy has been developed for adenovirus-based vaccines in the context of many diseases, and involves the incorporation of antigenic peptides within the capsid structure of adenovirus. Incorporating exogenous immunogenic peptides into the adenovirus capsid offers potential advantages, including a potent humoral response similar to the response generated by native adenoviral capsid proteins, and immune responses that can be boosted against antigenic epitopes with repeated administration (Matthews, 2010; Shiratsuchi et al., 2010; Roberts et al., 2006).

The adenoviral capsid is composed of three major proteins: hexon, fiber, and penton base. Hexon is the largest and most abundant capsid protein, with 720 copies per virion. Analysis of the protein sequences of different hexon proteins has revealed that there are seven discrete hypervariable regions (HVRs), which form the most exposed surface of the virion and are found to be the major targets of serotype-specific neutralizing antibodies (Crawford-Miksza and Schnurr, 1996; Gall et al., 1998; Rux et al.,

* Corresponding author. Tel.: +862034281614; fax: +8620 34281614.
** Corresponding author at: State Key Laboratory of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, 151 Yan Jiang Road, Guangzhou 510120, China. Tel.: +862034281614; fax: +8620 34281614.
E-mail addresses: 372546283@qq.com (C. Xue), tianxingui7902@aliyun.com (X. Tian), lixiao@gird.cn (X. Li), zzcmhxy003@163.com (Z. Zhou), xbsu92@yahoo.com (X. Su), zhourong@gird.cn (R. Zhou).
[1] These authors contributed equally to this work.

0168-1702/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2014.01.027

2003). The major capsid protein, hexon, has been used for antigen capsid-incorporation strategies because hexon plays a natural role in the generation of the anti-adenovirus immune response, and it is numerously represented within the adenoviral virion (Shiratsuchi et al., 2010; Krause et al., 2006; McConnell et al., 2006). Previous studies have verified that short heterologous peptides derived from poliovirus, *Pseudomonas aeruginosa*, *Bacillus anthracis*, and HIV, as well as model epitopes, can be incorporated into the adenoviral hexon HVRs without compromising viral viability (Worgall et al., 2007; Matthews et al., 2010; Crompton et al., 1994). For example, the replacement of HVR1 with a malarial B-cell epitope has been shown to induce a substantially increased level of protective humoral immunity against malaria and circumvents any preexisting immunity to adenovirus (Shiratsuchi et al., 2010).

The immune response against an epitope inserted into hexon is dependent on the incorporation site and the size of the incorporated epitope (McConnell et al., 2006; Wu et al., 2005). Published studies have focused on the incorporation of single epitopes or antigens into single HVRs. Recently, we sought to generate a multivalent vaccine Ad3 vector by incorporating epitopes in Ad3 hexon. However, our previous study showed that the antiserum was induced against the new epitope but not against the multiple epitopes that were simultaneously incorporated into single HVRs (Zhong et al., 2012). Therefore, the replacement of several HVRs with antigens might be a promising alternative way to generate multivalent adenoviral vectors. Our previous study confirmed that HVR1 and HVR2 of Ad3 are potential incorporation sites for vaccine development (Tian et al., 2012). The present study focuses on the creation of multivalent vaccine vectors displaying two different epitopes in several HVRs of Ad3.

2. Materials and methods

2.1. Cells, virus strains, and plasmids

Sublines of HEp-2 cells, AD293 cells, and Vero cells were kept in our laboratory and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, USA) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (Tian et al., 2012). The following plasmids and viruses used in this study were obtained as previously described or are maintained in our laboratory (Tian et al., 2012; Zhang et al., 2009): the E3-defective adenovirus type 3 replication-competent plasmid pBRAdV3dE3egfp (pAd3egf), which expresses the reporter molecule enhanced green fluorescent protein (EGFP) and the corresponding virus Ad3EGFP; hexon shuttle vector pBRHexonL/R; the plasmid pAd3egf-SP70 with the SP70 epitope in hexon HVR1 and the corresponding virus R1SP70A3 (R1A3); and the EV71-08-02 strain of the EV71 C4 genotype (GenBank accession no. FJ360545). The EV71 viral titer was determined as the $TCID_{50}$ in Vero cells, based on the typical cytopathic effect (CPE) produced by viral infection.

2.2. Recombinant hexon-modified plasmid construction

To generate constructs containing SP70 epitope in HVR1 and SP55 epitope in HVR2, HVR4, or HVR5, the SP55 and SP70 epitopes were genetically incorporated into the HVRs at the positions marked in Fig. 1. To achieve these genetic modifications, we first generated the hexon fragments containing the sequences encoding the SP70 and SP55 genes in different HVRs using an overlapping PCR method, as described previously by Tian et al. in 2012 (Tian et al., 2012). The corresponding primers are shown in Table 1.

The hexon fragments containing the SP70 and SP55 genes were purified and cloned into the Ad3 hexon shuttle vector pBRHexonL/R with *Cla*I and *Bam*HI restriction enzymes. To create recombinant Ad3 vectors containing the SP70 and SP55 sequences in the HVRs of hexon, the three shuttle vectors were digested with *Eco*RI and *Sal*I, and then used to cotransform *Escherichia coli* BJ5183 cells with the *Avr*II- and *Pac*I-linearized human adenovirus type 3 (HAdV3) plasmid pBRAdV3dE3egfp (pAd3egf). The resultant clones, which contained both SP70 and SP55, were obtained by homologous recombination, and the constructs were then selected with PCR using primers HexonF/sp55r or HexonF/sp70r (shown in Table 1) and confirmed by restriction digestion and sequence analysis.

2.3. Generation of recombinant virus

To rescue the recombinant virus, these modified plasmids were linearized with *Asi*SI. AD293 cells were transfected with 4 μg of each purified DNA using the NanoJuice Transfection Kit (Novagen, USA) and grown in dishes of 30 mm diameter. After the plaques formed, the viruses were processed for large-scale propagation in AD293 cells and then purified with CsCl gradient centrifugation (Wu et al., 2002). The purified viral DNA was confirmed with PCR and sequence analysis. The viral particle (VP) titers were determined with spectrophotometry using a conversion factor of 1.1×10^{12} VPs per absorbance unit at 260 nm.

2.4. Thermostability assay and growth characteristics

To test the heat stability of the hexon-modified adenovirus, the virus was incubated in DMEM containing 2% FBS at 45 °C for 0, 5, 10, 20, 40, or 60 min before it was used to infect HEp-2 cells. Fluorescence was then measured 48 h after infection with a Varioskan Flash Multimode Reader (Thermo Scientific), with excitation at 488 nm and recording the light emitted at 570 nm. Background fluorescence was normalized to wells containing cells only.

Growth curves were generated by infecting HEp-2 cells with adenovirus at five VPs/cell and the infected cells were collected every 12 h for 72 h. The harvested cells were suspended in DMEM containing 2% FBS, subjected to three freeze–thaw cycles, and centrifuged at 10,000 × *g* for 30 min at 4 °C to remove the cell debris. The viral suspension was then diluted with DMEM containing 2% FBS in a 10-fold dilution series and each dilution was used to infect HEp-2 cells cultured in 24-well plates. The number of infectious

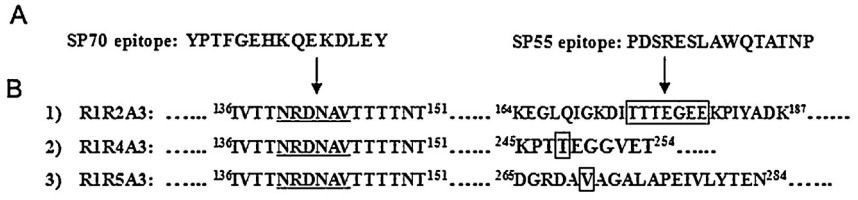

Fig. 1. Diagram of the SP55 and SP70 epitope incorporation sites in Ad3 hexon. (A) Amino acid residues of the SP70 and SP55 epitopes that were incorporated into the HVRs of Ad3 hexon. (B) R1R2A3, R1R4A3, or R1R5A3 corresponding to the hypervariable region (HVR) sites that were modified with the SP55 and SP70 epitopes. The underlined amino acid residues marked in HVR1 were replaced with the SP70 epitope, and the ones in the rectangles in regions 2, 4, and 5 were replaced with the SP55 epitope. The numbers show the positions of the amino acid residues in Ad3 hexon.