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Chimeric hexon HVRs protein reflects partial function of adenovirus

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ABSTRACT

Adenovirus is widely used in gene therapy and vaccination as a viral vector, and its hypervariable regions (HVRs) on hexon are the main antigen recognition sites of adenovirus. The modification of this area by genetic engineering will change the antigenic specificity of the virus. In addition, recent studies have demonstrated the importance of coagulation factor X (FX) in adenovirus serotype 5-mediated liver transduction in vivo. The binding site of adenovirus to FX is the HVRs on hexon. By constructing five proteins containing chimeric HVRs from different adenovirus serotypes, we focused on the antigenic specificity and the affinity for FX of these proteins compared with the corresponding viruses. Our data showed that HVR5 and HVR7 had only a part of hexon activity to neutralizing antibodies (NAbs) compared with the complete activity of HVR1–7. Results also demonstrated a differential high-affinity interaction of the HVRs proteins with FX and indicated that HVRs protein had a similar binding ability with corresponding adenovirus serotype. These results highlighted some properties of chimeric HVRs proteins and revealed the influence on the structure and function of hexon proteins and adenovirus resulting from the HVRs.

1. Introduction

Recombinant adenoviruses (rAds) have been used as vectors for several potential vaccines. Diseases for which adenovirus vectors are being considered include HIV, tuberculosis, malaria, and cancer [1]. A limitation that has become apparent with Ad5 (best-studied serotype) vaccine vectors is the high titers of Ad5 neutralizing antibodies (NAbs) in human populations, particularly in the developing world [2].

The adenovirus capsid consists of three major structural proteins: hexon, penton, and fiber. Sequence variability among adenovirus serotypes is concentrated on the several loops located at the solvent exposed surface of the hexon, termed hypervariable regions (HVRs). Previous studies showed that the dominant Ad5-specific NAbs are directed primarily against the hexon HVRs [3]. Hexon-chimeric rAd vectors, HVRs exchanges among adenoviruses from different virus subgroups, have been constructed and been proved to evade the majority of pre-existing anti-vector immunity in mice and rhesus monkeys. However, the final yields of these chimeric vectors were still lower than yields of the parental rAd5 vectors and some even have failed to rescue virus [3,4]. The relative importance of the seven individual HVRs as NAb epitopes also remains incompletely understood. Therefore, precisely mapping the NAb epitopes in these seven HVRs, such as HVR5 and HVR7, may be conducive to obtain more perfect chimeric rAd5 vectors [5]. However, construction of large amount of rAd vectors to address this are difficult to operate and expensive. For HVRs, some prior studies also demonstrated that human coagulation factor X (FX) binds the Ad5 hexon via an interaction between the FX Gla domain and HVRs leading to liver infection following intravascular delivery [6]. This specific interaction occurs in multiple (but not all) human adenovirus serotypes and shows diversities in the affinity. Similarly, which domains and amino acids in HVRs are integral to the high-affinity interaction with FX remains unclear [7].

In this study, we constructed and expressed several chimeric HVRs proteins and showed that the native proteins were oligomers and had consistent structure and function with that in virus. These results may provide useful insights for the future development of Ad-based vaccine and gene therapy.

2. Materials and methods

2.1. Cell lines and serum

Human embryonic kidney (HEK) 293, human lung epithelial A549, and human Chang liver cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Chang liver cells were cultured in RPMI 1640 medium. HEK293 and

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A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, (FBS; Hyclone; Logan, UT) at 37 °C in a humidified 5% CO_2. Anti-Ad5 and anti-Ad37 rabbit polyclonal serums were a gift from Dr. Panyong Mao (Beijing 302 Hospital, Beijing, China).

2.2. HVRs protein expression and purification

Sequences of HVRs from Ad5 and Ad37 were obtained from these two viral DNAs by PCR. The PCR was conducted with primers 5′-GATGAAGCTGCTACTGCTC and 3′-TTCATTTTTATCTGAAAATTCT, 5′-GACTACCAAAGAAAAGCAAAAC and 3′-TTGATTATGGGCTGAAATG, respectively. Sequences of Ad5HVR37(5,7), Ad5HVR37(1–7), Ad5HVR26(5,7), and Ad37HVR5(5,7) containing HVRs exchanged with the corresponding regions from Ad37, Ad26, and Ad5 were produced synthetically (Sangon biotech, shanghai, China). These sequences were then cloned into the pET20b plasmids (Novagen, Madison, WI) respectively by using a BamHI and an XhoI restriction site. Besides, a C-terminal His tag was contained in each of the plasmids. The purified plasmids were expressed in BL21 cells at 18 °C for 12–16 h to produce soluble HVRs proteins. The cells were collected by centrifuging at 4000 rpm for 30 min at 4 °C and resuspended with ice-cold 20 mM imidazole in a buffer containing 25 mM Tris–HCl (pH 8.0) and 500 mM NaCl, and then ultra-sonicated. After centrifugation at 12,000 rpm for 30 min at 4 °C, the supernatant containing HVRs proteins was purified by Ni–NTA beads (Qiagen, Valencia, CA) affinity chromatography. The proteins were eluted with 250 mM imidazole in the same buffer and analyzed by SDS–PAGE.

2.3. SDS–PAGE, gradually-denatured SDS–PAGE (GDS-PAGE) and Native-PAGE

Samples were denatured by boiling at 97 °C in Laemmli buffer (62.5 mM Tris–HCl, 2% SDS, 5% mercaptoethanol, 10% glycerol, 0.002% bromophenol blue) prior to SDS–PAGE. GDS–PAGE was basically similar to SDS–PAGE only except that the samples were not boiled in which the proteins were gradually denatured under the effect of SDS. Native-PAGE differed from SDS–PAGE in that the samples were mixed with a native buffer (62.5 mM Tris–HCl, 40% glycerol, 0.002% bromophenol blue) and were not denatured by boiling prior to electrophoresis performed on 6%–8% Tris–acetate gels (Invitrogen, Carlsbad, CA) without SDS contained in gels and running buffer.

2.4. Western blotting analysis

Protein samples were electrophoresed by SDS–PAGE, GDS–PAGE or Native-PAGE and transferred to nitrocellulose membranes for Western blotting. In particular, SDS was not added into the transfer buffer (39 mM glycine, 48 mM Tris–HCl, 20% methanol, 1.3 mM SDS, pH = 9.2) when proteins experienced Native-PAGE. The rabbit polyclonal serums as primary antibody were used at a 1/100 dilution and the ImmunoPure alkaline phosphatase-conjugated goat anti-rabbit IgG secondary Abs (Jackson ImmunoResearch Laboratories, Inc) were used at a 1/10,000 dilution. The color reaction was performed with 0.1 M Tris–HCl (pH 9.5) containing 0.66% NBT solution and 0.33% BCIP solution.

2.5. Viscotek gel permeation chromatography (GPC) analysis

Protein samples were loaded onto P4000 and P3000 columns using a Viscotek GPC/SEC system (Malvern, Worcestershire, UK) with triple detector platform (LS, RI, and VIS). Bovine serum albumin (BSA, 66 kDa) was used as the standard to calibrate the column. For each analysis, 100 ul of protein sample (1 mg/ml) was loaded and eluted with Dulbecco's Phosphate Buffered Saline (DPBS) at a flowrate of 1 ml/min.

2.6. Circular dichroism (CD) spectroscopy

The conformational integrity of the purified proteins was confirmed by CD spectrum to ensure their natural structures as expected. Far-UV CD spectra were obtained on a Jasco J-810 spectrophotometer (Tokyo, Japan) at 25 °C using a 0.5 cm path length. The solutions containing HVRs proteins were prepared with a concentration of 5 μM in 20 mM phosphate buffer solution (PBS) containing 100 mM NaCl, pH 8.0. The k2d was used to predict the secondary structure content of the proteins for CD spectrum.

2.7. Construction of HVRs chimeric rAd5 vectors

E1/E3-deleted, replication-incompetent rAd5 vectors containing chimeric hexon genes were constructed using a two-plasmid rescue method as described [8]. Partial Ad5 hexon genes containing the Ad5 HVRs exchanged with the corresponding regions from Ad37 or Ad26 were produced synthetically and cloned as ApaI-HpaI fragments into a shuttle plasmid containing the complete Ad5 hexon gene. The Ad5 HVR regions were defined as shown in Fig. 1A. AscI-AscI fragments containing the complete chimeric hexon genes were then excised from the shuttle plasmids and used to replace the corresponding regions in the Ad5 genome plasmid pBHGlox(delta)E1,3Cre. The resultant mutant Ad5 genome plasmids together with the small plasmid pDC316-EGFP expressing enhanced green fluorescent protein (EGFP) or pDC316-Luc expressing luciferase under control of a mouse CMV promoter were co-transfected into HEK293 cells, and homologous recombination yielded rAd5HVR37(5,7), rAd5HVR37(1–7), and rAd5HVR26(5,7) vectors. Plaque purification was performed to isolate a single clone as described [9]. The infectious titer (plaque forming units, PFU/ml) of the rAd vectors was determined by triplicate TCID50 assays using HEK293 cells.

2.8. Protein disrupt Ad5 neutralization assay (NA)

Ad5 NA was assessed by Luc-based virus neutralization assays as described previously [10]. A549 cells were plated at a density of 1×10^4 cells per well in 96-well plates. On the following day, serial dilutions of HVRs proteins with anti-Ad5 polyclonal antibody (Abcam, Cambridge, UK) or Ad5-seropositive rabbit serum were incubated for 1 h at 37 °C in 50 ul reaction volumes. Then a fixed amount of rAd5-Luc reporter constructs at a multiplicity of infection (MOI) of 500 were incubated for 1 h at 37 °C either alone (virus infection alone, VIA), which corresponded to 100% luciferase activity, or with the above protein-serum mixtures. As another positive control for virus replication, only virus with proteins (pAd37HVR) was added to cells, and as a negative control, cells were cultured in the absence of virus and virus with serum. Following 24 h incubation, luciferase activity in the cells was measured using the Steady-Glo Luciferase Reagent System (Promega, Madison, WI) with a Victor 1420 Multilabel Counter (Perkin Elmer, Wellesley, MA).

2.9. Adenovirus infection assay

HEK293 cells were grown in 6-well plates and were infected with the Ad vectors at an MOI of 10 with or without anti-Ad5 and anti-Ad37 rabbit serum. After incubation at 37 °C for 48 h, the fluorescent signal of EGFP was observed by fluorescence microscopy. The signal of the fluorescent proteins was detected at a low magnification (200×) by a fluorescence microscope. For luciferase expression, 2×10^4 Chang liver cells/well were plated