Microbial Pathogenesis 113 (2017) 421–426

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

Membrane-anchored stalk domain of influenza HA enhanced immune responses in mice

Dandan Gao[a,b], Yan Chen[a], Dong Han[a], Qiulan Qi[a], Xianliang Sun[a], Han Zhang[c], Hao Feng[a,*], Min Wang[a,**]

[a] Medical School of Jiaxing University, Jiaxing, Zhejiang 314001, China
[b] Jiaxing Maternity and Child Health Care Hospital, The Affiliated Hospital of Jiaxing University, Jiaxing, Zhejiang 314051, China
[c] Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA

ARTICLE INFO

Keywords:
Influenza virus
Stalk domain
(GGGGS)4 linkers
Virus-like particles
ADCC

ABSTRACT

Current strategies for influenza virus vaccines primarily aim to elicit immune responses towards the globular head domain of the hemagglutinin (HA) protein so that binding of the virus to membrane receptors on the host cells is inhibited. In the present study, we show a novel strategy to generate immunity against the highly conserved region of the influenza virus. The globular head domain was replaced by different linkers to generate a headless HA (stalk domain) and then coexpressed with influenza M1 proteinin Tni insect cells. The expression was validated by western blot analysis, and stalk domain with peptides (GGGGS)4 linkers was identified to anchor in a stable way to the cell membrane. An immunoelectron microscope showed that stalk domain with (GGGGS)4 linkers were steadily incorporated to the surface of influenza virus-like particles (VLPs). Mice immunized with these VLPs exhibited enhanced systemic antibody responses with increased binding avidity and study found high titers of ADCC antibodies to the influenza virus, these VLPs also induced mucosal immune responses and produced antigen-specific IgG and IgA in nasal and lung washes. In addition, antigen-specific IgG antibody-secreting cells (ASCs) increased significantly in the spleen and lymph node. The results of this study suggest that the headless HA is a useful target in developing a universal vaccine against influenza virus.

1. Introduction

Influenza (flu) is a serious public health problem worldwide and the flu vaccination is the best countermeasure against the viral infection. However, the effective influenza vaccines are limited because of the very narrow breadth of protection [1–3]. When the vaccine and circulating viral strains differ, the vaccine can provide very limited protection and there will be unexpected pandemic viruses. Thus, influenza vaccine development remains challenging.

Currently, highly conserved regions of viral proteins across a variety of subtype viruses are the main focus for the development of a universal vaccine against influenza. Among these regions, the stalk domain of hemagglutininis a specific target. Unlike the globular head domain of the HA protein, the stalk domain is relatively highly-conserved. It is composed of the N- and C-terminal parts of HA1, and the N-terminal part of HA2. The demarcation line between the stalk and head domain is defined by two cysteine residues that form a disulfide bond [4,5]. The stalk domain functions to mediate the fusion of viral and endosomal membranes once the virus is taken up into the cells, which is crucial for the release of the viral genome into the cytosol. As such, the stalk domain can be targeted by a broad spectrum of neutralizing antibodies, universal vaccine strategies, and perhaps also by antiviral drugs [6]. Indeed, for the development of a universal vaccine against influenza, various strategies use this feature to evoke immune responses toward this conserved region [7,8]. The influenza HA stalk domain lacking the globular head was first expressed successfully by Steel and colleagues who took advantage of the naturally occurring disulfide linkage between cysteines 52 and 277 [5]. They showed that mice vaccinated with this headless HA—HA stalk domain induced immune sera with broader reactivity than those obtained from immunized with native HA proteins [5]. Replacement of the globular head domain with a glycine linker resulted in a headless unstable HA protein expressed at low levels; the whole HA protein was collapsed without the globular head domain [6]. Hai et al. was later able to express a chimeric HA encompassing the H1 or H3 group stalk domain and the exotic head domain [4]. Vaccination with these constructs induces a high titer of stalk

* Corresponding author. Jiahang road 118#, Nanhu District, Jiaxing city, Zhejiang province, 314001, China.
** Corresponding author. Jiahang road 118#, Nanhu District, Jiaxing city, Zhejiang province, 314001, China.
E-mail address: fenghao0550@sina.com (H. Feng).

https://doi.org/10.1016/j.micpath.2017.11.025
Received 8 July 2017; Received in revised form 17 November 2017; Accepted 18 November 2017
Available online 21 November 2017
0882-4010/

reactive antibodies in mice and ferrets and elicits broadly protective stalk-specific antibodies [9]. Bommakanti et al. used a protein design and minimization method for successful expression and refolding of HA2 into its neutral pH conformation, the designed HA2-based immunogen was shown to be highly immunogenic in mice [8], engineering of structurally-intact "headless" HA constructs have also been reported to preserve the critical epitope bound by broadly-neutralizing antibodies [10,11].

Virus-like particles (VLPs) are an effective platform for increasing the immunogenicity of antigens [12]. The icosahedral structure features the virus particles repetitive array of their component proteins, particulate nature, and ability to appropriately stimulate an innate immune response [13]. In this study, we generated a construct with the influenza stalk domain and replaced the globular head domain with a peptide linker (GGGGS)4, or an alpha-helical rigid peptides (EAAAK)4, two peptide linkers used extensively in fusion protein expression [14,15]. This headless HA (stalk domain) with part of HA2 and HA1 was then coexpressed with M1 to produce influenza VLPs. The immunogenicity of the generated VLPs was tested in mice.

2. Materials and methods

2.1. Cells

Fall armyworm(*Spodoptera frugiperda*) Sf9 cells (Sf9, ATCC, CRL-1711) were maintained in serum-free SF900 II medium (GIBCO-BRL, Grand Island, NY) at 25–27 °C. Tni ovarian cells isolated from the cabbage looper moth (*Trichoplusia ni*) (Tni) were maintained in ESF921 in spinner flasks at a speed of 80–100 rpm.

2.2. Construction of membrane-anchored stalk domain

The HA and M1 gene were from PR8 sequence, the headless HA constructs were generated by fusion PCR methods. Briefly, the head domain was replaced by the peptide linker (GGGGS)4 or the α-helical peptides (EAAAK)4, and a GCN4PII sequence-stabilized trimeric stalk domain (mHA) construct was generated. To generate the gene encoding the membrane-anchored mHA, the coding sequence of SUMOstar [16] and murine CD59 GPI anchor were fused to the 5′- and 3′- ends of the mHA coding gene, followed by GCN4PII and His tag sequence. This full-length construct encoding a GPI-anchored mHA was confirmed by DNA sequencing. The gene encoding membrane-anchored stalk domain and M1 protein were cloned into pFastbac1.

2.3. Cell surface expression of membrane-anchored mHA

Recombinant baculovirus (rBVs)-expressing mHA and M1 were generated by Bac-to-Bac expression system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Insect cells Sf9 for production of rBVs and Tni cells were used to express protein. The presence of membrane-anchored mHA on insect cell surfaces was determined by a cell surface immune-assay. Briefly, 2 days post infection with mHA rBV, Tni cells were incubated with NHS-SS-biotin dissolved in PBS (0.5 mg/mL) for 30 min at 4 °C. Biotinylation was quenched by adding PBS containing 0.1 N glycine. The cells were lysed and precipitated with avidin resins (Fisher Scientific Inc., Rockford, IL) at 4 °C overnight. The resins were then washed with lysis buffer plus 0.4% SDS and mixed with loading buffer (125 mM Tris-HCl pH7.5 containing 4% SDS, 20% glycerol, plus 10%-mercaptoethanol) and heated at 95 °C for 5 min. The samples were separated on 10% SDS gel and transferred to a nitrocellulose membrane, then probed with HA antiserum dilution.

2.4. Generation of influenza VLPs

Tni cells were co-infected with rBVs expressing influenza mHA and M1, respectively [17]. 48 h post infection, the culture supernatant was collected and VLPs were concentrated by porous fiber filtration using the Quixstand benchtop system (GE Healthcare, Uppsala, Sweden) followed by sucrose density gradient ultracentrifugation. To quantitate the yield of purified VLPs, the protein concentration of each sample was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The samples were separated on 10% SDS gel and western blot with HA antiserum to detect the expression of mHA in pelleted VLPs.

2.5. Cell surface immunogold labeling and electron microscopy of infected cells

Infected Tni cells were blocked in HEPES-1.5% BSA after washed with HEPES containing lysine. The cells were then incubated with mouse anti-HA antibody (Bioss Inc., Beijing, China) at a 1:100 dilution at 4 °C for 1 h. After washing, a 10 nm gold-conjugated goat anti-mouse antibody (Sigma-Aldrich, St. Louis, MO) was added at a dilution of 1:100 for 1 h. Cells were then washed and fixed with 1% glutaraldehyde in PBS for 45 min, incubated with 1% osmium tetroxide for 1 h, and then stained with tannic acid. The samples were dehydrated and embedded in EMBED 812 (Electron Microscopy Sciences, Hatfield, PA). Samples were then stained with uranyl acetate and lead citrate and examined with a Philips CM 10 electron microscope.

2.6. Animal immunization and sampling

The animal experiments were conducted with prior approval from the Animal Welfare andEthics Committee of Jiaxing University. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Jiaxing University (Permit Number: JUMC2016-003).

Female 3–4 weeks old BALB/c mice (HFK Biotechnology, Beijing, China) were immunized with an immunization regimen including one intramuscular (i.m.) prime and followed by two intranasal (i.n.) boosts with 50 μg soluble mHA or mHA VLPs at 3-week intervals. Blood were collected by retro-orbital plexus puncture. Sera were collected by a spin at 5000 rpmafter clotting. Nasal washes were collected by lavaging mouse nostrils repetitively with 200 μl of PBS containing 0.05% Tween 20 (PBST). For lung washes, individual mouse lungs were lavaged repetitively with 0.5 mL PBST. After centrifugation at 5000 rpm, supernatants were filtered through a 0.22 μm filter and stored at −80 °C for further assays. Lymphocytes from node and spleen samples collected from mice sacrificed 4 weeks after the final boost were used for ELISPOT.

2.7. Antibody ELISA

HA-specific antibody (Ab) titers in immune sera were determined by ELISA as described previouslyusing mHA as coating antigens. The highest dilution (giving an OD 450 at least twice the standard deviation of the control group at the same dilution) was designated as the Ab endpoint titer.

2.8. Antibody avidity and ADCC(antibody-dependent cell-mediated cytotoxicity, ADCC) assay

Avidity assay: 96-well microtiter plates were coated with HA protein at 400 ng/well at 4 °C overnight, the diluted serum samples were added to wells and incubated for 2 h at 37 °C. The assay was conducted using parallel titrations of immune sera with or without treatment with 1.5 M sodium thiocyanate (NaSCN) for 15 min to discriminate weak binding from high-affinity binding between antibodies and HA protein, following the binding of immune sera. The antibody titers were subsequently determined using the standard ELISA procedure. The avidity index (AI) was calculated by dividing the titer with NaSCN treatment by the titer without the NaSCN treatment and multiplying by 100 [18].

ADCC assay: the chromium-release assay were used to calculate the