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Cross-clade protective immune responses of NS1-truncated live attenuated H5N1 avian influenza vaccines

Shaohua Shi[a,b,c,d], Sujuan Chen[a,b,c], Weizhou Han[a,b,c], Bai Wu[a,b,c], Xiaojian Zhang[a,b,c], Ying Tang[a,b,c], Xiao Wang[a,b,c], Yinbiao Zhu[a,b,c], Daxin Peng[a,b,c,*], Xiufan Liu[a,b,c]

[a] College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu, PR China
[b] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, Jiangsu, PR China
[c] Jiangsu Research Center of Engineering and Technology for the Prevention and Control of Poultry Disease, Yangzhou, Jiangsu, PR China
[d] Institute of Animal Husbandry and Veterinary Medicine, Fujian Academy of Agricultural Sciences, Fuzhou, Fujian, PR China

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ABSTRACT

Background: H5N1 highly pathogenic avian influenza (HPAI) has raised global concern for causing huge economic losses in poultry industry, and an effective vaccine against HPAI is highly desirable. Live attenuated influenza vaccine with trunctated NS1 protein as a potential strategy will be extremely useful for improving immune efficacy.
Methods: A series of H5N1 avian influenza virus reassortants harboring amino-terminal 48, 70, 73, and 99 aa in NS1 proteins, along with a modified low pathogenic HA protein was generated, and named as S-HALo/NS48, S-HALo/NS70, S-HALo/NS73, and S-HALo/NS99, respectively. In addition, their biological and immunological characteristics were further analyzed.
Results: The viruses S-HALo/NS70, S-HALo/NS73, and S-HALo/NS99, but not S-HALo/NS48, had a comparable growth property with the full-length NS1 virus, S-HALo/NSFu. Mice and chickens studies demonstrated that the viruses with truncated NS1 protein were further attenuated when compared to the virus S-HALo/NSFu. Vaccination with the virus S-HALo/NS73 in chickens induced significant cross-protection against homologous clade 2.3.4 H5 virus and heterologous clade 7.2, 2.3.2.1, and 2.3.4.4 H5 viruses.
Conclusion: A 70-aa amino-terminal fragment of NS1 protein may be long enough for viral replication. The recombinant virus S-HALo/NS73 is a broad-spectrum live attenuated H5N1 avian influenza vaccine candidate in chickens.

1. Introduction

H5N1 highly pathogenic avian influenza (HPAI) is not only an acute, highly contagious fatal respiratory disease in poultry, resulting in significant economic losses in poultry industry, but also poses a severe public health threat and may cause a potential influenza pandemic. After undergoing a series of evolutionary, multiple clades (clades 7.2, 2.3.2.1 and 2.3.4.4) and multiple NA subtypes (H5N1, H5N2, H5N5, H5N6, and H5N8) of H5 viruses have co-circulated in China recently [1–5].

Although stamping-out program against HPAI has been carried out in most developed countries, vaccination combined with the culling strategy may be considered to be an effective tool to prevent the spread of HPAI H5N1 viruses in developing countries [6]. Nowadays in China, inactivated vaccines were widely used, including several reassortant avian influenza vaccines, such as vaccine Re-4 to Re-7, which not only elicit strong humoral response, but also match the antigenic diversity of prevalent H5N1 strains. However, it is commonly accepted that no adequate mucosal or cellular immunity is achieved by this kind of inactivated vaccine [7]. A distinct advantage of live virus vaccines is that they may elicit long-lasting, broader immune (humoral and cellular) response, because they closely mimic the natural infection. Historically, live attenuated virus vaccines are available through temperature-sensitive (*ts*) mutation [8–10] and cold-adaptation [11]. Recently, another concern of live attenuated virus vaccines was focused on modification of NS1.

During the viral life cycle, the NS segment of influenza A virus encodes two proteins, NS1 and NS2, through alternative splicing of its mRNA [12]. NS1 protein has been shown to counteract the

* Corresponding author at: Yangzhou University, College of Veterinary Medicine, 48 East Wenhui Road, Yangzhou, Jiangsu 225009, PR China. Tel.: +86 514 87979224; fax: +86 51487979386.
E-mail address: pengdx@yzu.edu.cn (D. Peng).

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host type I interferon (IFN) response [13–15]. It can also inhibit adaptive immunity via attenuating the innate immune function of dendritic cells [16]. A mutant influenza virus lacking NS1 protein is shown to be highly attenuated in interferon-competent cells and hosts [17], and live attenuated influenza vaccine can be achieved by suitable truncation of NS1 protein. Several effective vaccine candidates have been developed for mouse [18,19], swine [20,21], equine [22], macaque [23], and chicken [24,25].

In this study, we developed a panel of NS1-truncated variants and evaluated their replication characteristics and immune protection against H5 subtype HPAIV. The results showed that S-HALo/NS73 had been adequately attenuated with strong protection against homologous and heterologous H5 HPAIV challenge, indicating that it could be accepted to be a suitable live attenuated H5N1 avian influenza vaccine candidate in chickens.

2. Methods

2.1. Cells and viruses preparation

Human embryonic kidney (293T) and Madin-Darby canine kidney (MDCK) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. The NS1 donor H5N1 strain, A/Mallard/Huadong/S/2005 (S; clade 2.3.4, vaccine strain Re-5-like, GenBank accession numbers: EU195389-EU195396) [26] and the challenge H5 viruses A/Chicken/Huadong/4/2008 (H5N2) (DT, clade 7.2, vaccine strain Re-7-like) [27], A/Duck/Jiangsu/JY03/2012 (H5N1)(WX; clade 2.3.2.1, vaccine strain Re-6-like), A/Goose/Changzhou/CZG/2014 (H5N8) (CZG; clade 2.3.4.4) were propagated in specific-pathogen-free (SPF) embryonated chicken eggs.

2.2. Construction of plasmids for expressing modified HA and truncated NS1

To generate attenuated influenza viruses, the HA gene was modified through removing the multibasic cleavage site and inserted into the pHW2000 vector to construct recombinant plasmid pHW-HALo [27]. The NS1 gene was truncated by the strategy as described in a previous study [24]: deletions and stop codons were introduced into NS1 gene segment, resulting in a series of truncated-NS1 proteins harboring only 48, 70, 73, or 99 amino acids in the amino terminus, whereas no change was done in NS2 gene. Each truncated NS gene was inserted into the pHW2000 vector and the recombinants were named as pHW-NS48, pHW-NS70, pHW-NS73, or pHW-NS99, respectively.

2.3. Virus rescue

Virus rescue was performed as previously described [28] with some modifications. Briefly, the co-cultured 293T-MDCK cells were transfected with rescue plasmids (pHW-PB2, pHW-PB1, pHW-PA, pHW-HALo, pHW-NP, pHW-NA, pHW-M, and pHW-NS) [26], with/without the substitution of plasmids pHW-NS48, pHW-NS70, pHW-NS73, or pHW-NS99 using Polyfect Transfection Reagent (Qiagen, GmBH, Germany). Meanwhile, the plasmid of pHW-HA [26] was substituted for pHW-HALo to rescue NS1-truncated viruses for the purpose to determine whether NS1 deletions were sufficient to attenuate a virus carrying a highly pathogenic H5 HA. At 48 h post-transfection, the cell supernatant was then inoculated into SPF eggs to amplify the recombinant viruses, and the genomic sequence of the fifth passage recombinant virus was sequenced to confirm the correction of the rescued viruses.

2.4. Quantification of IFN-β mRNA using real-time RT-PCR

Chicken embryo fibroblast (CEF) cells were infected with the recombinant viruses at a multiplicity of infection (MOI) of 0.001 in the presence of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin. At 24 h post-infection, total RNA was extracted and reverse transcribed, followed by IFN-β quantitation by real-time PCR in a LightCycler system (LightCycler®Nano, Roche, USA). The β-actin mRNA was amplified as an internal control to normalize cellular RNA levels for sample to sample variations using standard procedures. The relative IFN-β mRNA levels compared to that of the mock-infected CEF cells were calculated using the threshold cycle $2^{-\triangle\triangle CT}$ method [29].

2.5. Growth curves of NS1-truncated viruses in CEF cells and embryonated chicken eggs

Cultures of CEF cells were infected with each recombinant virus at an MOI of 0.001 in the presence of exogenous TPCK-treated trypsin. At 0, 12, 24, 36, and 48 h post-inoculation, the titer of each virus in culture supernatant was determined by $TCID_{50}$ assay. Next, groups ($n = 18$) of SPF chicken eggs were inoculated with the recombinant viruses at a dose of 10^2EID_{50}. Allantoic fluid from three inoculated eggs was harvested at 0, 12, 24, 36, 48, and 60 h post-inoculation and subsequently assayed by EID_{50}.

2.6. Virulence in BALB/c mice

Six-week-old female BALB/c mice (~20 g) were anesthetized prior to intranasal infection ($n = 5$) with 10^6 EID_{50} of the recombinant viruses in 50 μl PBS or mock-infected. The mortality, weight loss, or other disease signs of mice were recorded daily in a 2-week period. The 50% mice lethal dose (MLD_{50}) was calculated by the Reed–Muench method [30]. For the viral replication kinetics in mouse model, mice were inoculated with recombinant viruses through the same route described above, and three mice in each group were euthanized on days 3 and 6 post-inoculation. The livers, spleens, lungs, kidneys, and brains were sampled for virus titration by EID_{50} assay.

2.7. Infection in SPF chickens

The intravenous pathogenicity index (IVPI) test was conducted to assess the pathogenicity of recombinant viruses carrying NS70, NS73, NS99, and wild-type NSFu along with HA or modified HA (HALo) according to the recommendation of the OIE [31].

To detect the viral replication kinetics in chickens, 2-week-old white SPF chickens were inoculated intranasally with 10^6 EID_{50} of each virus and monitored daily. On days 3 and 6 post-inoculation, four chickens per group were euthanized and their hearts, livers, spleens, lungs, kidneys, brains, and pancreases were sampled for virus titration through EID_{50} assay.

2.8. IFN-β mRNA levels in the lungs of infected SPF chickens

To detect the IFN-β mRNA expression *in vivo*, 2-week-old white SPF chickens were inoculated intranasally with 10^6 EID_{50} of each virus, and the lungs of infected chickens were collected on days 3, 6, and 14 post-infection. The viral titers in the lungs were determined by EID_{50} assay and the relative levels of IFN-β mRNA were determined by real-time RT-PCR.

2.9. Vaccination and homologous challenge

The two-week-old white SPF chickens ($n = 10$) were intranasally vaccinated with 10^6EID_{50} recombinant viruses in a 100-μl volume