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Human 4F5 single-chain Fv antibody recognizing a conserved HA1 epitope has broad neutralizing potency against H5N1 influenza A viruses of different clades

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ABSTRACT

Influenza A viruses present a significant threat to public health worldwide. High-affinity human scFv antibodies against a conserved epitope can potentially provide immunity to diverse viruses and protect against future pandemic viruses. A library of phage-displayed human scFv containing 6.0×10^8 members was generated from lymphocytes of H5N1 virus vaccinated individuals. Using the recombinant H5N1 virus hemagglutinin ectodomain (HA1), 4F5 scFv was identified with neutralizing activity against both clade 2 and 9 H5N1 viruses. In embryonated chicken eggs, the antiviral activity of 4F5 scFv conferred a 100% survival rate and at least a 62.5% survival rate against different clades of H5N1 viruses by pre-treatment and post-treatment, respectively. 4F5 scFv belongs to the VH-3-43 family according to the IMGT database, and a peptide (76)WLLGNP(81) containing half of an α-helix in HA1 was identified as the binding pocket. The conserved binding epitope of this novel broadly neutralizing scFv may become key in the design and implementation of vaccines or RNA interference against H5N1 viruses.

1. Introduction

Influenza A viruses have caused four human pandemics in the last century and still pose threats to public health (Gregg et al., 1978; Kilbourne, 2006; Oxford, 2000; L'vov et al., 2004). Infection and transmission in humans by the highly pathogenic H5N1 avian flu in the past few years (Andrade et al., 2009; Olsen et al., 2005; Ungchusak et al., 2005; Wang et al., 2008), particularly in Southeast Asian countries (Kandun et al., 2006; Chotpitayasunondh et al., 2005), with either high mortality or morbidity have heightened fear that the next influenza pandemic is due (Doerr et al., 2006; Ebrahim, 2004). Mutation of the viral genome due to antigenic drift and shift (Russell and Webster, 2005; Shih et al., 2007; Matrosovich et al., 2000), especially in the hemagglutinin (HA) and neuraminidase (NA) genes, necessitates the development of prophylactic and therapeutic interventions that can provide broad protection.

Vaccination undoubtedly is the principal strategy for prevention and control of influenza (Subbarao et al., 2006; Chen et al., 2008; Nakaya et al., 2011). However, the current vaccines have limitations in that they can only protect those at risk from the viruses circulating at the time of development (Sasaki et al., 2011; Tang et al., 2008). Another possible effective countermeasure against influenza is antibody-based therapy (Lu et al., 2006; Nguyen et al., 2010). The resurgence of this type of therapy has been fueled by the reports of individuals surviving severe influenza infection after transfusion with convalescent plasma (Kong and Zhou, 2006). Of the 3 major surface glycoproteins on the virion, HA is the primary target for neutralizing antibodies. HA is a glycoprotein, composed of HA1 and HA2 subunits, and three HA monomers form a homo-trimer to function in receptor binding by HA1 (Chiu et al., 2009) and membrane fusion by HA2. HA has been classified into 16 antigenically distinct subtypes, and it is generally believed that neutralizing antibodies are not cross-reactive among HA subtypes. However, the identification of monoclonal antibodies with broad, heterosubtypic neutralizing activity (Sakabe et al., 2010; Ohshima et al., 2011; Ekiert et al., 2009; Hultberg et al., 2011; Oh et al., 2010; Wrammert et al., 2011; Clementi et al., 2011) suggests that such conserved epitopes in HA do exist.

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Recently, a binding pocket was characterized on HA for the fusion inhibitor tert-butyl hydroquinone (Sui et al., 2009), which shows great promise as a new target for therapy. The HA2 domain is rich with α-helices, which can form hydrophobic pockets and facilitate binding with antibodies. Antibodies binding to these concave pockets have been reported to neutralize different clades or even subtypes of influenza A viruses. There are also three α-helices in the HA1 domain, but antibodies against these sites with cross-reactivity to other viruses have not been previously reported.

In this study, we isolated and characterized a human single-chain Fv (scFv) antibody, designated 4F5, with neutralizing activity against different clades of H5N1 influenza A virus. It bound to a conserved peptide (76)WLLGNP(81) in the HA1 domain and showed satisfactory antiviral effects against challenge with H5N1 viruses in embryonated chicken eggs. Altogether, our findings indicate that the scFv may be developed for use as prophylaxis or treatment of influenza A virus infections.

2. Materials and methods

2.1. Viruses

The viruses used in this study were: A/chicken/Hongkong/369/2003 (H5N1) (GenBank: KC784945.1), A/goose/Jilin/514/2005 (H5N1) (GenBank: KC784947.1), A/Jiangsu/4/2007 (H5N1) (GenBank: KC784948.1), A/goose/Guangdong/08/2005 (H5N1) (GenBank: KC784944.1), A/Jiangsu/6/2008 (H5N1) (GenBank: KC784946.1) and A/Jiangsu/1/2007 (H5N1) (GenBank: EU434686.1). The whole-virion H5N1 vaccine was manufactured in embryonated hens' eggs using the reassortant strain NIBRG-14 (A/Vietnam/1194/2004-A/PR/8/34) as vaccine virus. The A/Vietnam/1194/2004 virus belongs to clade 1, and the inactivated vaccine was developed by the Beijing Sinovac Biotech Co. Ltd. and China Center for Disease (Qiu and Yin, 2008).

2.2. Expression of H5N1 recombinant HA1 (rHA1) protein in a Bac-to-Bac baculovirus expression system

Total RNA was isolated from A/Jiangsu/1/2007 (H5N1), and HA1 was amplified with H5-specific primers. The amplified products were subcloned into pFastBac and transformed into *Escherichia coli* DH10Bac. The positive recombinant bacmid was isolated and transfected into Sf9 cells, as described previously (Li et al., 2010). Recombinant HA1 proteins were detected with a horseradish peroxidase (HRP)-conjugated anti-His antibody. An anti-H5N1 virus mouse monoclonal antibody (mAb) affinity chromatography column was used to purify the rHA1 protein.

2.3. Construction of scFv antibody library

Lymphocytes were isolated from healthy donors from the Chinese Han population who were immunized with whole-virion H5N1 vaccines. Written informed consent was obtained from each volunteer. V_H and V_L genes were amplified by RT-PCR using a group of specific constant region primers (Barbas et al., 2004). ScFv fragments were spliced by overlapping PCR and ligated into pComb3XSS phagemid. A scFv phage display library was constructed after the ligations were introduced into competent *E. coli* XL1-Blue by electroporation. The library was rescued and amplified using VCSM13 helper phage.

2.4. Selection of rHA1-specific clones from the scFv library

For phage bio-panning, rHA1 was coated onto ELISA plates. Amplified phage mixtures were then incubated with the coated wells, and the unbound phage were removed by washing with PBST. Bound phage were eluted with glycine–HCl, pH 2.2. Eluted phage were neutralized to pH 7.0, followed by infection of *E. coli* XL1-Blue. After three rounds of selection, the phage were amplified, plated on agar plates and incubated overnight. Single bacterial colonies were picked and tested by phage-ELISA.

The scFv coding regions of the positive colonies were amplified and sequenced. Their framework regions (FRs) and complementary determining regions (CDRs) were determined by the Kabat system (Kabat et al., 1979). Amino acid sequence alignments of the V_H regions and V_L regions of the scFv antibodies were performed to compare with the human immunoglobulin sequences (IMGT Database) (Lefranc, 2011; Ehrenmann et al., 2010).

The positive scFv-phagemids were extracted from *E. coli* XL1-Blue colonies and transformed into non-suppressor *E. coli* Top10F'. These clones were cultured for large-scale soluble scFv production, and HisTrap™ chromatography was used to purify the scFv proteins.

2.5. Microneutralization assay

Neutralizing antibody titers were determined by microneutralization assays performed on Madin–Darby canine kidney (MDCK) cells, following a previously described procedure (Hu et al., 2008). The 50% tissue culture infective dose ($TCID_{50}$) was calculated by the Reed and Muench method (Krah, 1991; Reed and Muench, 1938). Briefly, serially diluted scFv antibodies were incubated with 100 $TCID_{50}$ of virus before adding to the MDCK cells. Plates were incubated for 40 h before fixation of the cells with acetone. The presence of viral protein was detected with an anti-NP antibody. The concentration for 50% of maximal effect (EC_{50}) of the antibody was expressed as the lowest concentration of scFv antibodies giving 50% neutralization of 100 $TCID_{50}$ of virus in MDCK cells. Anti-CTGF mAb and PBS were used as controls.

2.6. Hemagglutination inhibition (HI) assay

In a microtiter plate, a series of 2-fold dilutions of the H5N1 virus (50 μl per well) were incubated at 37 °C for 1 h with an equal volume of 0.5% chicken erythrocytes. Hemagglutination (HA) titers were calculated.

In another microtiter plate, a series of 2-fold dilutions were made with antibody (25 μl per well), and an equal volume (25 μl) of virus suspension (diluted to contain 4-HA units per 25 μl) was added to each well. After incubation for 1 h, 50 μl of 0.5% erythrocyte was added and incubated at 37 °C for 1 h. The HI titers were expressed as the lowest concentration of scFv antibodies that completely inhibited hemagglutination. Anti-CTGF mAb and PBS were also used as controls.

2.7. Western blotting and immunofluorescence assay

Inactivated viruses were separated by SDS–PAGE and detected with the scFv by Western blotting. Immunofluorescence assays were used for analysis of scFv binding to native HA proteins in MDCK cells infected with H5N1 viruses. MDCK cells were infected with H5N1 viruses separately until the cytopathic effect reached 60–70%.The cells were then fixed and incubated with the scFv antibody. The mouse anti-His antibody and Alexa Fluor555-labeled goat anti-mouse antibody were added to the cells in succession. After DAPI staining, the cells was observed by using a laser scanning confocal microscope.