Permutation of M2e affects the effectiveness of universal influenza nanovaccine

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Abstract

Influenza A virus (IAV), a deadly zoonotic pathogen, occasionally cross-species transmission among humans, swine and avian. The ectodomain of matrix protein 2 (M2e) is highly conserved in IAV and has been widely concerned in the development of universal influenza vaccines. Due to low immunogenicity, multi-copy M2e are usually displayed on the surface of nanoparticles to constitute universal nanovaccines. Here, we report that the permutation of the M2e affects the immune effect of the nanovaccine. Three M2e derived from humans, swine and avian IAV were inserted into the C-terminal of the Cap protein of porcine circovirus type 2 (PCV2) to form self-assembled nanovaccine. Immunoprotective effects of different M2e arrangements were explored in mice. Results showed that the M2e closest to the surface of nanoparticle induced the most efficient protection against IAV derived from corresponding species. The results will help in the development of more effective universal influenza vaccines, especially for specific species.

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Running title: Universial influenza nanovaccines

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Summary

Influenza A virus (IAV), a deadly zoonotic pathogen, occasionally cross-species transmission among humans, swine and avian. The ectodomain of matrix protein 2 (M2e) is highly conserved in IAV and has been widely concerned in the development of universal influenza vaccines. Due to low immunogenicity, multi-copy M2e are usually displayed on the surface of nanoparticles to constitute universal nanovaccines. Here, we report that the permutation of the M2e affects the immune effect of the nanovaccine. Three M2e derived from

humans, swine and avian IAV were inserted into the C-terminal of the Cap protein of porcine circovirus type 2 (PCV2) to form self-assembled nanovaccine. Immunoprotective effects of different M2e arrangements were explored in mice. Results showed that the M2e closest to the surface of nanoparticle induced the most efficient protection against IAV derived from corresponding species. The results will help in the development of more effective universal influenza vaccines, especially for specific species.

Keywords: influenza A virus; porcine circovirus type 2; M2e; universal; nanovaccine; permutation

1 Introduction

Influenza A virus (IAV) causes huge economic loss to the global husbandry industry and poses a significant threat to public health. The IAV genome is composed of eight single-stranded negative-sense RNA fragments, resulting in abnormally high frequency of gene mutations and recombination, which bring great difficulties to the development of universal vaccines (Lowen, 2017). IAV occasionally cross species boundaries and pose lethal threats to other species (Long, Mistry, Haslam, & Barclay, 2019). In particular, cross-species transmissions between humans, swine and birds are more frequent, such as 2009 pandemic H1N1 swine influenza, highly pathogenic H5N1 and H7N9 avian influenza jumped into humans, causing great panic (Gao, 2018). Therefore, it is urgent to establish IAV universal vaccines.

Ectodomain of matrix protein 2 (M2e) is the most conservative and protective viral antigen and can generate hetero-subtype immunity against multiple virus strains and subtypes. However, M2e is difficult to be recognized by the immune system due to the low molecular weight, the low abundance and the steric blocking by HA and NA on the surface of IAV (Kolpe, Schepens, Fiers, & Saelens, 2017). Various effective strategies for improving M2e antibody levels have been proposed, in particular, some nanoparticle-based nanovaccines show exciting immune effects, such as ferritin, virus-like particles (VLPs), and gold nanoparticles(Kolpe et al., 2017). To further improve the level of M2e-specific antibodies, nanoparticles usually display multi-copy of M2e. Tandem expression of M2e from human, swine and avian IAV are the most common pattern (Deng, Chang, et al., 2018; Deng, Mohan, et al., 2018; Ding, Jin, Chen, et al., 2019; K. H. Kim et al., 2018; M. C. Kim, Lee, et al., 2013; M. C. Kim et al., 2015; M. C. Kim, Song, et al., 2013; Petukhova et al., 2013; Qi et al., 2018; Y. Wang et al., 2020; Yong, Yeap, Ho, Omar, & Tan, 2015). However, due to the uncertainty of the crystal structure of M2e and immune systems tend to efficiently recognize protruding domains on the surface of nanoparticles, it is worth investigating whether the permutation of M2e of IAV from different species has an impact on the immune effect of nanovaccines.

In this research, we displayed the M2e derived from IAV of humans, swine and avian in different order (six orders) at the C-terminal of porcine circovirus type 2 (PCV2) Cap VLPs to explore the effect of different M2e arrangement on the immune effect of the universal vaccine. Results showed that the M2e closest to surface of Cap VLPs induced the highest M2e-specific antibodies and conferred the best protection against IAV of corresponding species. This result will help to develop more efficient universal influenza vaccines.

2 Methods

2.1 Expression and purification of recombinant proteins

Triple M2e peptides derived from human (hM2e), swine (sM2e) and avian (aM2e) IAV were combined with the C-terminus of Cap protein in different arrangements using Gly-Gly-Gly-Gly linker. (Figure 1a-1c). The cysteine of M2e were mutated to serine. These sequences were inserted into the pET28a vector by BamHI and HindIII digestion enzymes, and transformed into *E. coli* BL21 (DE3). Then these transformed cells were induced expression at 20°C for 15 h by isopropyl-β-d-thiogalactoside (IPTG) (0.2 mM). These recombinant proteins were purified by using Ni-NTA His·Bind Resin and identified by SDS-PAGE and Western blot. The concentrations of these purified proteins were determined with a BCA protein assay kit. The endotoxin concentrations were measured by ToxinSensor Single Tests Kit.

2.2 Particle characteristics of these Cap-3M2e VLPs

These Cap-3M2e proteins were dialyzed into the assembly buffer (10 mM Tris-HCl, 100 mM NaCl (pH 8.0)) and formed VLPs. The shape, size, size distribution and zeta potential of these Cap-3M2e VLPs were characterized by transmission electron microscopy (TEM) and dynamic light scattering (DLS).

2.3 Immunization and challenge

Female BALB/c mice (6-8 weeks old) were randomized into groups. Six groups were subcutaneously immunized with 26.6 μg (100 μL) candidate vaccines, respectively. The Cap VLPs group (20 μg, Cap molar equivalent) is serves as positive control. The Carbopol 971P as adjuvant (Ding et al., 2017). Booster immunization was performed at the 21 and 42 days post initial immunization (dpi). Serum samples were collected at 63 dpi and stored at -20 °C until use. Mice in each group were divided into three groups (n=9). Mice were lightly anesthetized and intranasally challenged with lethal dose humans, swine and avian IAV at 64 dpi, respectively. Survival rates and body weight loss were monitored daily for 14 days post challenge (dpc). Mice were humanely euthanized if weight loss of [?] 25%.

2.4 Antibody titer detection

M2e- and PCV2-specific antibodies in sera were determined by indirect ELISA using 1 μg/mL of synthesized M2e peptides as coating antigens. Twofold serially diluted immune serum samples were added into the ELISA plates, followed by HRP-conjugated goat anti-mouse IgG. The highest dilution which showing over twofold OD450 readout than that of the control sample as the antibody endpoint titer. For the detection of PCV2-specific antibodies, commercial PCV2 antibody test kit (BioChek, Reeuwijk, Holland) was utilized.

2.5 Determination of IAV titers

Four mice from each group were sacrificed at the time when the virus titers were highest in the lungs. The MDCK cell-based immunoperoxidase monolayer assay was used for lung virus titration as described elsewhere (Ding, Jin, Zhou, et al., 2019).

2.6 Ethics Statement

All BALB/c mice received humane care and all animal procedures in this study were performed in accordance with the Institutional Animal Care and Use Committee (IACUC).

2.7 Statistical analysis

All statistics were performed using GraphPad Prism (ver 7.0) (GraphPad Software, San Diego, CA, USA). Quantitative data are reported as means ± SEM. P values were determined by one-way ANOVA analysis. Statistical significance was determined at $p < 0.05$ (*).

3 Results

3.1 Characteristics of Cap-3M2e VLPs

Six different recombinant Cap-3M2e proteins were expressed in *E. coli* and purified using $Ni_2{}^+$-NTA column. SDS-PAGE and Western blot showed that these recombinant Cap-3M2e proteins were successfully expressed and purified (Figure 1d). These recombinant Cap-3M2e proteins can be recognized well with the anti-PCV2

polyclonal antibody and 14C2 monoclonal antibody (Anti-IAV M2 protein), indicating that these recombinant proteins retained the reactogenicity of M2e and the Cap protein (Figure S1). Endotoxin levels in these recombinant proteins were less than 0.18 EU/mg.

TEM images showed that these recombinant Cap-3M2e proteins could self-assemble into VLPs (Figure 1e and Figure S2). DLS test results showed that the diameter of these Cap-3M2e VLPs were larger than that of Cap VLPs (Figure 1f). The zeta potential of these Cap-3M2e VLPs were lower than that of Cap VLPs, which were caused by the strong negative charge of M2e molecules (Figure 1g). The particle size and surface charge of these Cap-3M2e VLPs are consistent, indicating that the permutation of M2e of IAV from different species does not affect the morphology and surface charge of these VLPs (Figure 1f and 1g).

3.2 Humoral immune effects

All Cap-3M2e VLPs groups induced high levels of total M2e-specific antibodies (Figure S3a). There were significant differences in levels of antibodies against M2e derived from human, swine and avian IAV in sera of each group. The Cap-hsaM2e VLPs and Cap-hasM2e VLPs groups induced higher anti-human IAV M2e antibodies than others (Figure 2a). Anti-human IAV M2e antibody in the Cap-hasM2e VLPs group was 7.3 times that of the Cap-sahM2e VLPs and Cap-ashM2e VLPs groups (Figure 2a). The Cap-shaM2e VLPs and Cap-sahM2e VLPs groups induced higher anti-swine IAV M2e antibodies than other groups (Figure 2b). Anti-swine IAV M2e antibody in the Cap-shaM2e VLPs group was 6.4 times that of the Cap-ashM2e VLPs group (Figure 2b). Meanwhile, anti-avian IAV M2e antibodies in the Cap-ahsM2e VLPs and Cap-ashM2e VLPs were the highest (Figure 2c). The anti-avian IAV M2e antibody in Cap-ahsM2e VLPs group was 8 times that of the Cap-sahM2e VLPs group (Figure 2c). However, the PCV2-specific antibodies in all groups were consistent, indicating that the insertion of three copies of M2e in different orders did not affect the immunogenicity of the Cap VLPs (Figure S3b).

3.3 Protective efficacy of Cap-3M2e VLPs against IAV

All mice immunized with Cap VLPs died by 4 to 6 dpc with the highest virus titers in lungs and over 25% body weight loss (Figure 2d-2l). The protective efficacy of groups Cap-hsaM2e VLPs and Cap-hasM2e VLPs were the highest among all groups, and protected all mice from lethal infection when challenged with 10 $\times$ LD_{50} A/Puerto Rico/8/1934 (H1N1) (Figure 2d-2f). Similarly, the Cap-shaM2e VLPs and Cap-sahM2e VLPs groups showed the highest protection efficacy, and conferred complete protection against 10 $\times$ LD_{50} of A/swine/Zhucheng/90/2014 (H1N1) (Figure 2g-2i). The Cap-ahsM2e VLPs and Cap-ashM2e VLPs groups demonstrated the lowest body weight loss, mortality rate and lung virus titers among all groups, when challenged with 40 μL 10^9 $TCID_{50}$/mL of A/chicken/Guangzhou/GZ/2005 (H9N2) (Figure 2j-2l).

4 Discussion

The M2e is a highly conserved candidate epitope in different subtypes of IAV and offers potential to develop universal vaccines, if it can be appropriately presented and sensed by host immune system. Displaying multiple copies of M2e molecules on the surface of nanoparticles is an effective means to increase the level of anti-M2e antibodies. Although M2e is conserved among IAV, there are still some differences among strains. Particularly, M2e derived from different species varies considerably (Schepens, De Vlieger, & Saelens, 2018). Therefore, nanoparticles usually display M2e of human, swine and avian IAV in tandem to increase the broad spectrum.

Affinity maturation of antibodies requires stable and full display of epitopes rather than transient state (Dormitzer, Ulmer, & Rappuoli, 2008). However, M2e adopt at least two transformed conformations (Cho et al., 2016; Cho et al., 2015). In addition, due to the existence of flexible links between different M2e, the

instability of M2e conformation is exacerbated. Therefore, the relatively stable one in the multiple copies of M2e is more easily recognized by the immune system and eventually induces higher levels of antibodies.

VLPs, as a kind of nanoparticles with precisely defined three-dimensional structure, provide a large number of sites for M2e that can be accurately inserted (Rodriguez-Limas, Sekar, & Tyo, 2013). The N-terminal, C-terminal and loops are usually the prominent sites of VLPs suitable for insertion into M2e. However, loops are generally flexible structures and can only tolerate peptides of limited length (D. Wang et al., 2018). In this study, the C-terminal of Cap protein protrudes from the surface of Cap VLPs, and participates in the formation of linear and conformational neutralization epitopes, indicating that the C-terminus can be efficiently recognized by immune system (Khayat et al., 2011; Lekcharoensuk et al., 2004). Therefore, it can be utilized as an insertion site for multiple copies of M2e. Results showed that the M2e which near the C-terminal induced higher levels of species-specific anti-M2e antibodies. It was because the immune system efficiently recognized the C-terminal of Cap protein, thereby efficiently recognizing adjacent M2e. The level of species-specific anti-M2e antibodies induced by two M2e that away from the C-terminal are low and there no significant difference. This is due to the indefinite spatial conformation of M2e and the wobble of the flexible link leading to the decline of the immune system's recognition ability.

Therefore, it is necessary to selectively display M2e of IAV of species-specific in the most prominent and relatively fixed position of nanoparticles based the immune target of universal IAV vaccines, so as to induce a more efficient immune effect. For example, when chickens inject with universal IAV nanovaccines, M2e of the avian IAV need to be displayed in a prominent and stable position on nanoparticle.

Conclusion

In this study, we studied the effect of the permutation of three M2e peptides derived from human, swine and avian IAV on the surface of PCV2 VLPs on the immune effect of IAV universal vaccine. Results demonstrate that the M2e peptide closest to the surface of the nanoparticle induced the highest immune protection against this species of IAV. This result suggests that the M2e of IAV of a specific host should be stably displayed when designing universal nanovaccines.

Acknowledgments

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Conflict of interest

The authors report no conflicts of interest are associated with this work.

Data availability statement

Data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure 1 Characterization of these Cap-3M2e VLPs.

(a) Primary pattern structure of six recombinant Cap-3M2e. (b) M2e sequence of the human, swine and avian IAV which were used in challenging experiment. (c) Schematic illustration of Cap-3M2e VLPs nanovaccine. The VLPs model was derived from the PDB database (3R0R). (d) SDS-PAGE analysis of these purified Cap-3M2e proteins. Lane M: molecular weight markers; lane 1: Cap; lane 2: Cap-hsaM2e; lane 3: Cap-hasM2e; lane 4: Cap-shaM2e; lane 5: Cap-sahM2e; lane 6: Cap-ahsM2e; lane 7: Cap-ashM2e. (e) Transmission electron micrograph (TEM) of Cap-3M2e VLPs. The image is part of the TEM of Cap-ashM2e VLPs. Scale bars = 50 nm. (f) Size distribution of these Cap-3M2e VLPs (n=5). (g) Zeta potential of these Cap-3M2e VLPs (n=5).

Figure 2 Protective efficacy of these Cap-3M2e VLPs against IAV.

(a) Human IAV M2e-specific IgG level (n=6). (b) Swine IAV M2e-specific IgG level (n=6). (c) Avian IAV M2e-specific IgG level (n=6). (d) Mice body weight changes (n=5), (e) survival rate (n=5) and (f) lung virus titers (n=4) post 10 × LD_{50} of A/Puerto Rico/8/1934 (H1N1) challenge. (g) Mice body weight changes (n=5), (h) survival rate (n=5) and (i) lung virus titers (n=4) post 10 × LD_{50} of A/swine/Zhucheng/90/2014 (H1N1) challenge. (j) Mice body weight changes (n=5), (k) survival rate (n=5) and (l) lung virus titers (n=4) post 40 μL 10^9 $TCID_{50}$/mL of A/chicken/Guangzhou/GZ/2005 (H9N2) challenge. $p < 0.05$ (*).