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Cell-Penetrating Peptide Penetratin as a Potential Tool for Developing Effective Nasal Vaccination Systems

Keiya Muto [1], Noriyasu Kamei [2], Mia Yoshida [1], Kozo Takayama [1], Mariko Takeda-Morishita [2,*]

[1] Department of Pharmaceutics, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan
[2] Laboratory of Drug Delivery Systems, Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe, Hyogo 650-8586, Japan

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ABSTRACT

Nasal vaccination is considered an attractive strategy to prevent the infection and spread of viruses. However, the vaccine formulations available on the market remain imperfect on account of their limited effectiveness. In the present study, we hypothesized that the nasal coadministration of antigens with cell-penetrating peptides promotes antigen delivery immune response in the nasal mucosa, thereby enhancing the production of mucosal IgA and systemic IgG. The levels of ovalbumin (OVA)-specific IgG and IgA in plasma and nasal perfusate, respectively, increased after 2 or 4 weeks on nasal coadministration of OVA with L- or D-penetratin, suggesting that OVA antigen was effectively delivered by penetratin to the nasal epithelium. An additional study demonstrated that the production of systemic IgG and nasal mucosal IgA against influenza A virus was specifically promoted by nasal coadministration of influenza A virus with D-penetratin. The results of this study suggested that cell-penetrating peptides are a promising tool for the delivery of vaccines to the nasal mucosa and for the subsequent dual stimulation of systemic and mucosal immune responses.

Introduction

Systemic injections are the main route for vaccine administration and are only capable of inducing the production of IgG in the blood, whereas they cannot generate IgA in common infection sites such as respiratory and gastrointestinal mucosal tracts.[1,2] Therefore, generating IgA in the mucosal surfaces is critical to prevent viral infections.[3,4] Nasal vaccination is an ideal alternative to systemic injections on account of its ability to generate systemic IgG and mucosal IgA and its excellent usability.[3,5-7] Although it is now possible to use attenuated live vaccines such as FluMist® (which have been approved in the United States, but are currently unauthorized in Japan), there are concerns over their limited immunization efficiency in adults and possible virulence. To obtain effective immune responses on nasal vaccination, delivery technologies have to be developed that can boost the uptake of antigens and adjuvants by nasal epithelial immune cells.[8]

Our recent publications clearly demonstrated that cell-penetrating peptides (CPPs) such as highly cationic oligoarginines[9,10] and the amphipathic peptide penetratin from the *Drosophila* Antennapedia homeodomain[11] could dramatically increase the nasal absorption of peptide- and protein-based biopharmaceuticals.[12-14] Moreover, we have shown that CPPs-based stimulation of the nasal absorption was mediated by noncovalent intermolecular interactions between the drug and CPP and by the multiple modes of energy-dependent and energy-independent cellular internalization pathways.[15] We therefore hypothesized that the noncovalent strategy using CPPs can also be applicable to the mucosal antigen delivery for the development of the nasal vaccines. In the present study, we tested the ability of penetratin (the most promising CPP) to increase the internalization of ovalbumin (OVA) and influenza virus type A and the resultant generation of specific IgG and IgA in mouse plasma and nasal mucosa, respectively.

Materials and Methods

Materials

L-penetratin (RQIKIWFQNRRMKWKK, capital letters indicate the L-form of amino acids) and D-penetratin (rqikiwfqnrrmkwkk,

The authors Keiya Muto and Noriyasu Kamei contributed equally.

* *Correspondence to:* Mariko Takeda-Morishita (Telephone: +81-78-974-4826; Fax: +81-78-974-4820).
E-mail address: mmtakeda@pharm.kobegakuin.ac.jp (M. Takeda-Morishita).

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lowercase letters indicate the D-form of amino acids) were synthesized by Sigma-Genosys, Life Science Division of Sigma-Aldrich Japan Co. (Hokkaido, Japan). OVA was purchased from Sigma-Aldrich Co. (St. Louis, MO). Influenza virus type A strain Texas/1/77 H3N2 was purchased from Acris Antibodies, Inc. (San Diego, CA), which contains the purified fraction from amniotic fluids of eggs infected with influenza virus strain Texas 1/77 (H3N2; hemagglutinin activity: 38,000 units/mL). All other chemicals were of analytical grade and are commercially available.

Preparation of OVA or Influenza A Virus Penetratin Mixed Solutions

Specific amounts of OVA and L- or D-penetratin were separately measured in polyethylene tubes and dissolved in phosphate-buffered saline (PBS, pH 7.4) containing 0.001% methylcellulose, which prevents the adsorption of the peptides and proteins onto the tube surface. Influenza A virus stock solution (0.89 mg/mL) was diluted with PBS supplemented with 0.001% methylcellulose. Next, OVA or influenza A virus and L- or D-penetratin solutions were added together to a final concentration of 0.5 mg/mL (OVA and influenza A virus) and 0.5 or 2 mM (penetratin) and mixed gently.

Immunization Protocols

Female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and used at 6-9 weeks of age. Animals were housed in controlled rooms at 23 ± 1°C and 55 ± 5% relative humidity, and they had free access to water and food during acclimatization. This research was performed at Hoshi University and complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals. Mice were coadministered intranasally with a 10-μL aliquot (5 μL/each nostril) containing 5 μg of OVA or influenza A virus and L-penetratin (2 mM) or D-penetratin (0.5 or 2 mM) at days 0, 7, 14, and 21.

Collection of Plasma and Nasal Perfusate Samples

One week after the second (day 7) and final (day 21) immunization, 0.8 mL of blood was taken from the heart using heparinized syringes, and the nasal cavity was washed with 1 mL of PBS containing 1% bovine serum albumin (BSA), and perfusate was collected. Plasma was separated by centrifugation at 13,400 × *g* for 1 min. In the study using influenza A virus, plasma and nasal perfusate were only collected 1 week after the final immunization.

Measurement of Antigen-Specific Antibodies by ELISA

Antigen-specific antibody levels in plasma (IgG) and nasal perfusate (IgA) were determined using ELISA. First, OVA (1 mg/mL) or influenza A virus (5 μg/mL) in PBS was incubated in microtiter plates (Bethyl Laboratories, Inc., Montgomery, TX) overnight at 4°C to be immobilized onto the plate surface, and the plates were blocked with PBS containing 1% BSA for 1 h at room temperature. Plasma and nasal perfusate were serially diluted in PBS with 1% BSA and 0.05% Tween 20 and incubated overnight at 4°C with coating antigens. After washing with PBS containing 0.05% Tween 20, the appropriate concentration (1:4000 dilution in PBS with 0.05 % Tween 20) of horse radish peroxidase–conjugated goat antimouse IgG- or IgA-specific antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) was added, and the plates were incubated for 1 h at room temperature. After washing with PBS containing 1% BSA and 0.05 % Tween 20, TMB solution prepared by diluting TMB Peroxidase Substrate (Bethyl Laboratories, Inc.) with Peroxidase Substrate Solution B (Bethyl Laboratories, Inc.) was incubated for 2 min at room temperature. The reaction was terminated by the addition of 0.5 N HCl, and then absorbance was measured at 450 nm.

Statistical Analysis

Each value is expressed as mean ± standard error of the mean of 3-6 determinations. The significance of the differences in the mean values of multiple groups was evaluated using ANOVA with Dunnett's test. The IBM SPSS Statistics Version 22 (IBM Corp., Armonk, NY) was used for statistical analysis. Differences were considered significant when the p value was less than 0.05.

Results and Discussion

Stimulation of IgA and IgG Generation Via Nasal Coadministration of OVA With Penetratin

Our recent work strongly suggested that CPPs could be an attractive tool for boosting the uptake/permeation of macromolecular peptides and protein drugs into/through the epithelial membranes.[12,13,15] Furthermore, we found that penetratin was the most promising CPP, which can deliver the peptide and protein drugs effectively and safely into the nasal and intestinal epithelial cells.[12,14,16] Therefore, in this study we tested whether antigens nasally coadministered with penetratin could be delivered into nasal epithelial immune cells to produce IgA and over the epithelial barriers into systemic circulation to produce IgG.

Figures 1a and 1b show OVA-specific IgG and IgA generation after nasal coadministration of OVA with L-or D-penetratin. The levels of anti-OVA antibodies in plasma and nasal perfusate at day 7 (2 and 4 weeks) after the second and last nasal immunizations with OVA suggest that 2-mM D-penetratin induced higher anti-OVA plasma IgG and mucosal IgA levels than 2-mM L-penetratin.

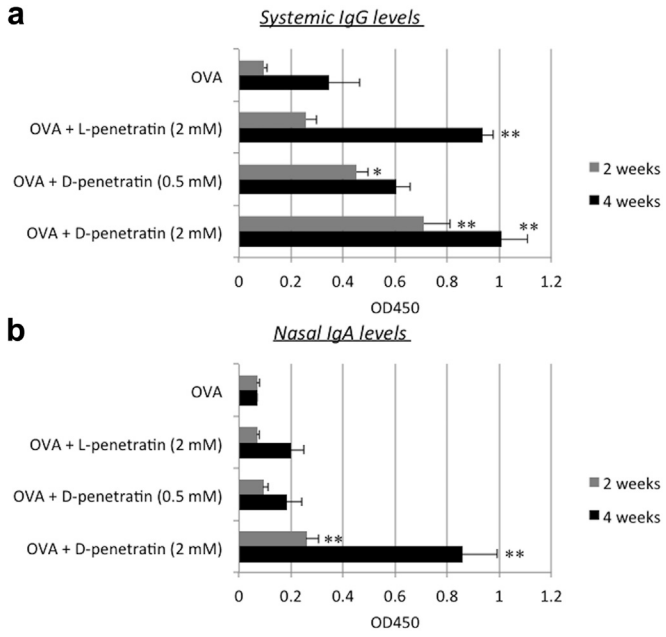

Figure 1. Levels of OVA-specific immunoglobulins in systemic and nasal mucosa after nasal coadministration of OVA with L- or D-penetratin (0.5-2 mM) at days 0, 7, 14, and 21. Panels (a) and (b) represent the levels of systemic IgG and nasal mucosal IgA, respectively. Plasma and nasal perfusate samples were collected at days 14 and 28 and diluted 16,384 times and 4 times, respectively, before ELISA measurements. Data are presented as mean ± standard error of the mean (n = 3-6). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. OVA group (control).