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The safety of a mucosal vaccine using the C-terminal fragment of *Clostridium perfringens* enterotoxin

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The C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) is a claudin-4 binder. Very recently, we found that nasal immunization of mice with C-CPE-fused antigen activated antigen-specific humoral and mucosal immune responses and that the deletion of the claudin-4-binding domain attenuated the immune responses. C-CPE-fusion strategy may be useful for mucosal vaccination. C-CPE is a fragment of enterotoxin, and the safety of C-CPE-fused protein is very important for its future application. In the present study, we investigated whether C-CPE-fused antigen induces immune responses without mucosal injury by using ovalbumin (OVA) as a model antigen. Immunohistochemical analysis showed that claudin-4 was expressed in epithelial cell sheets bordering the nasal cavity. Nasal immunization with C-CPE-fused OVA dose-dependently elevated the OVA-specific serum IgG titer, which was 1000-fold greater than the titer achieved by immunization with OVA or a mixture of OVA and C-CPE at 5 μg of OVA. Nasal immunization with C-CPE-fused OVA (5 μg of OVA) activated Th1 and Th2 responses. Histological analysis showed no mucosal injury in the nasal cavity or nasal passage. C-CPE-fused OVA exhibited mucosal vaccination without mucosal injury. These findings indicate that claudin-4-targeting using C-CPE can be a potent strategy for mucosal vaccination.

1. Introduction

Vaccination is the most potent therapeutic method to overcome infectious diseases. Vaccines are classified as parenteral or mucosal. Parenteral immunizations activate systemic immune responses, while mucosal immunizations activate both systemic and mucosal immune responses. Parenteral vaccination can activate immune responses against the invaded pathogenic microorganisms and infected cells; in contrast, mucosal vaccination prevents entry of the pathogenic microorganisms and activates immune responses against the infected cells (Kunisawa et al. 2008; Neutra and Kozlowski 2006). Although mucosal immunization is promising, an immune response is not activated by the mucosal administration of antigen alone. Efficient antigen delivery into mucosal associated lymphoid tissue (MALT) is the key technology needed for the development of mucosal vaccines (Kunisawa et al. 2008; Neutra and Kozlowski 2006).
The mucosa is covered by epithelial cell sheets, which separate the outside of the body from the inside of the body. Tight junctions (TJs) are located between adjacent epithelial cells and seal intercellular junctions, preventing the free movement of solutes across epithelial cell sheets (Scheneeberger and Lynch 1992). Claudin, a tetra-transmembrane protein family consisting of 24 members, plays a pivotal role in the mucosal TJ barrier (Furuse and Tsukita 2006; Tsukita et al. 2001). In 2003, Tamagawa et al. reported that claudin-4 is expressed in the epithelium of intestinal MALT. These findings indicate that claudin-4-targeting may be a novel strategy for the development of mucosal vaccines; however, the claudin-4-targeting vaccine had never been developed because of a delay in the preparation of claudin-4 binder.
Clostridium perfringens enterotoxin (CPE) causes food poisoning in humans (McClane and Chakrabarti 2004). The CPE receptor is claudin-4, and a 14-kDa polypeptide, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), is a claudin-4 binder (Katahira et al. 1997; Sonoda et al. 1999). We previously found that C-CPE enhanced jejunal, nasal and pulmonary absorption of drugs through its interaction with claudin-4 (Kondoh et al. 2005; Uchida et al. 2010). C-CPE is used as a claudin-4 ligand molecule for proteins (Ebihara et al. 2006; Saeki et al. 2009). These findings strongly indicate that C-CPE may be a potent ligand for MALT. Very recently, we have found that intranasal administration of C-CPE-fused ovalbumin (OVA) increased OVA-specific immune-responses in serum, nasal, vaginal and intestinal mucosa (Kakutani et al. 2010). However, C-CPE is a fragment of enterotoxin, and the safety of C-CPE-fused vaccine has never been investigated.
In the present study, we investigated whether nasal immunization with C-CPE-fused OVA activated immune responses without mucosal injury, and we found that mucosal vaccine using C-CPE activated Th1 and Th2 immune responses without nasal mucosa injury.

2. Investigations and results

C-CPE is a binder of claudin-4, and we recently found that claudin-4-targeting using C-CPE might be a potent strategy for mucosal vaccine (Kakutani et al. 2010). C-CPE is a fragment

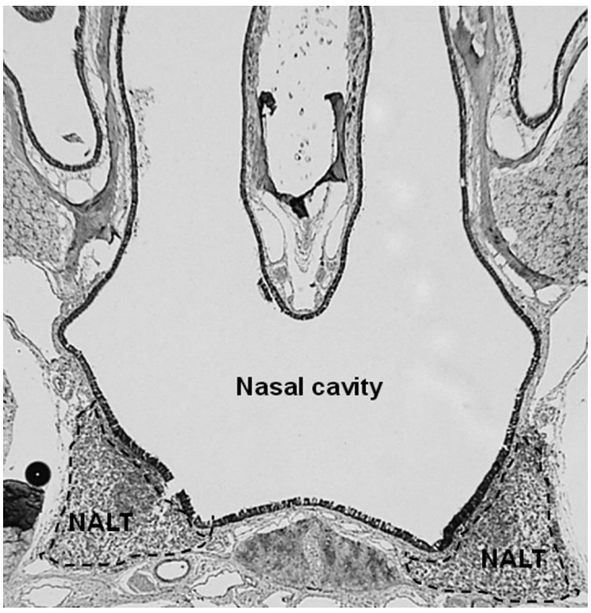


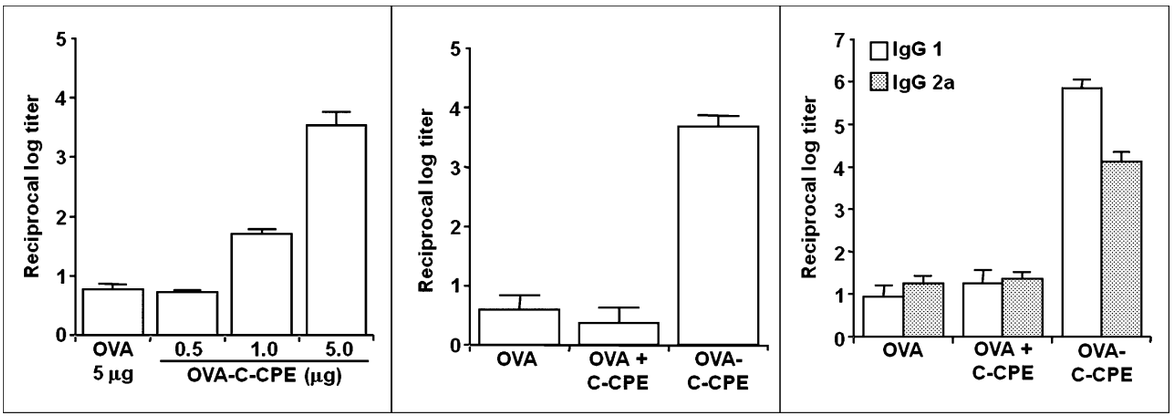
Fig. 1: Immunohistochemical analysis of claudin-4 in NALT Specimens were cryosectioned (4 μm) and stained with anti-claudin-4 Ab, followed by horseradish peroxidase-labeled secondary Ab. The immunoreactive regions were stained with a commercially available staining kit. The regions surrounded by dotted lines are NALT

of CPE (Katahira et al. 1997). CPE is a 35-kDa single polypeptide toxin produced by *Clostridium perfringens*. Experimental and epidemiologic evidence indicates that CPE causes foodborne disease and non-foodborne diarrheal illnesses (McClane 2001; McClane et al. 2000). CPE forms a CPE-containing complex in the plasma membrane that creates massive alterations in plasma membrane permeability that lead to cell death and histological damage to the intestine (McClane and Chakrabarti 2004). The safety of C-CPE is a critical issue for pharmaceutical applications of C-CPE. First, we investigated the expression of claudin-4 in nasal mucosa. Immunohistochemical analysis reveals that claudin-4 is expressed in the epithelium bordering the nasal cavity (Fig. 1). The epithelium that covers nasal MALT, nasopharynx-associated lymphoid tissue (NALT), is rich in claudin-4. These data correspond to our previous data on the expression of claudin-4 mRNA and protein in NALT (Kakutani et al. 2010).

To investigate the dose dependency of OVA-C-CPE in mucosal vaccination, mice were nasally immunized with OVA-C-CPE at 0.5, 1.0 or 5.0 μg of OVA. As shown in Fig. 2A, OVA-specific serum IgG levels were elevated in a dose-dependent manner and reached a level that was 1000-fold greater than the OVA values at 5 μg of OVA, which is the maximal dose of OVA-C-CPE due to its solubility. A mixture of OVA and C-CPE did not increase OVA-specific serum IgG levels (Fig. 2B), and OVA-C-CPE (5.0 μg of OVA) immunization activated IgG2a (a Th1 immune response) and IgG1 (a Th2 immune response) responses (Fig. 2C). We performed a histopathological analysis of mice immunized with OVA-C-CPE at 5.0 μg of OVA. Hematoxylin and eosin (HE) staining revealed no apparent mucosal injury in the nasal squamous cavity, the respiratory cavity, and the nasal passage (Fig. 3A). There was also no inflammatory cell infiltration in the nasal mucosa (Fig. 3B). These findings indicate that nasal immunization with OVA-C-CPE activated immune responses without histological injury in nasal mucosa.

3. Discussion

Only a mucosal vaccine can prevent the entry of pathological viruses into the mucosal membrane; however, injectable vaccines are currently used in both developing and industrialized countries. Very recently, we found that claudin-4-targeting using C-CPE can be a novel strategy for mucosal vaccination (Kakutani et al. 2010). In the present study, we showed that nasal immunization with C-CPE-fused antigen activated Th1 and Th2 immune responses without histological injury in nasal mucosa. The efficient delivery of C-CPE-fused antigen to immunocompetent cells is critical for mucosal vaccination. A mixture of OVA and C-CPE did not activate immune responses, and therefore OVA may be delivered to the immunocompetent cells as the C-CPE-fused protein. The uptake of nasally administered antigens is achieved through a unique set of antigen-sampling cells, the M cells, located in follicle-associated epithelium. After the uptake of antigens by M cells, the antigens are immediately processed and presented to the underlying dendritic cells (Neutra and Kozlowski 2006). A recent report indicates that claudin-4 is expressed in M cells (Rajapaksa et al. 2010). Claudin-4 contains sorting signal sequences to endosomes, an ALGVLL motif at amino acids 92 to 97 and a YVGW motif at amino acids 165 to 168 (Ivanov et al. 2004). These findings suggest that OVA-C-CPE may be taken up by clathrin-mediated endocytosis in



(A) (B) (C)

Fig. 2: Production of OVA-specific serum IgG by nasal immunization with OVA-C-CPE Mice were nasally immunized with OVA (5 μg), a mixture of OVA (5 μg) with C-CPE or OVA-C-CPE at the indicated dose of OVA once a week for 3 weeks. Seven days after the last immunization, the serum IgG level was determined by ELISA (A). Mice were nasally immunized with OVA, a mixture of OVA with C-CPE or OVA-C-CPE at 5 μg of OVA once a week for 3 weeks. Seven days after the last immunization, the serum IgG (B), IgG1 and IgG2a (C) levels were determined by ELISA. Data are means ± SEM (n = 4)

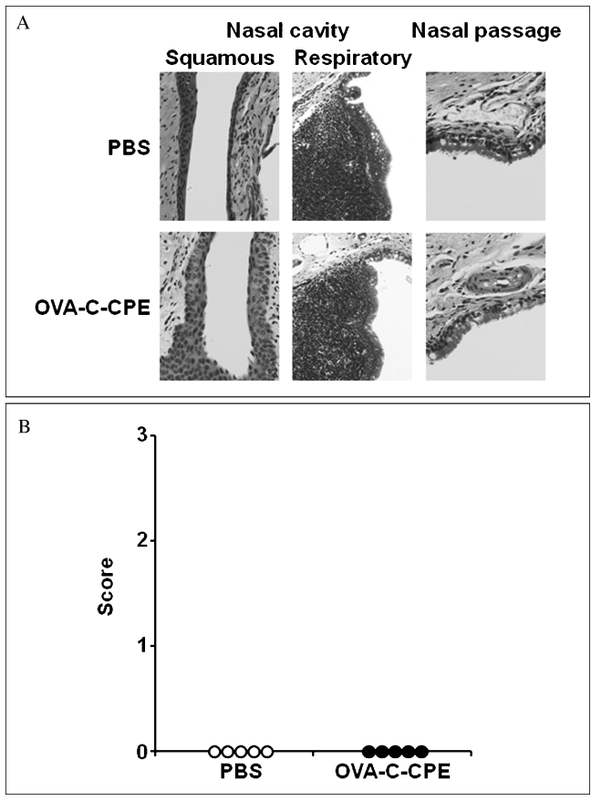


Fig. 3: A lack of histological injury caused by OVA-C-CPE Mice were nasally immunized with PBS or OVA-C-CPE (5 μg OVA) once a week for 3 weeks. Seven days after the last immunization, NALT was collected and fixed with formalin. Thin tissue-sections were stained with hematoxylin (A), and inflammation was scored according to the severity of the inflammatory cell infiltration (B): 0, none; 1, weak; 2, moderate; 3, severe. Scoring was performed blindly to avoid bias

M cells. The underlying mechanism for immunopotentiation by OVA-C-CPE must be clarified.

Nasal mucosa is adjacent to the central nervous system, and intranasal drug delivery to the brain has been developed (Dhuria et al. 2010). An influx of a vaccine protein into the central nervous system can be a risk factor in its clinical application. C-CPE is a modulator of the epithelial barrier. Treatment of nasal mucosa with C-CPE increased the nasal absorption of a peptide drug. OVA-C-CPE also modulated the epithelial barrier *in vitro* (data not shown). Claudin-deficient mice showed a size-dependent leak of solutes smaller than 1,000 Da in the epithelium and endothelium (Furuse et al. 2002; Nitta et al. 2003). C-CPE enhanced the mucosal absorption of dextran with a molecular mass of ∼20 kDa (Kondoh et al. 2005). Because OVA-C-CPE has a molecular mass of 65 kDa, and the infiltration of inflammatory lymphocytes was not observed in nasal mucosa (Fig. 3B), OVA-C-CPE might not cause an influx of solutes across the nasal epithelium. The claudin family comprises at least 24 members. Interestingly, the barrier-function and expression profiles of claudin family members differ among tissues. Claudin is believed to form homophilic and heterophilic adhesions in TJ strands, and various combinations of the 24 family members are thought to create diversity in the structure and functions of TJ barriers (Furuse and Tsukita 2006; Morita et al. 1999; Tsukita et al. 2001). Targeting the type of claudin specifically expressed in NALT may reduce the risk of delivering solutes to the central nervous system.

In summary, we showed that nasal immunization with C-CPE-fused antigen activated Th1 and Th2 immune responses without mucosal injury. This is the first report to indicate the safety of a claudin-4-targeting mucosal vaccine using C-CPE. Future improvement of the claudin specificity may lead to clinical applications of this type of vaccine.

4. Experimental

4.1. Animals

Female BALB/c mice were purchased from SLC, Inc. (Shizuoka, Japan). The mice were housed at 23 ± 1.5 °C with a 12-h light/dark cycle and had free access to standard rodent chow and water. The protocol of this study was approved by the Animal Care and Use Committee for Graduate School of Pharmaceutical Sciences, Osaka University.

4.2. Immunohistochemical analysis

Immunohistochemical staining for claudin-4 was performed with an autostainer (Dako, Glostrup, Denmark). Slide-mounted, fixed cryosections (4-μm thick) of nasal MALT were incubated in Dako target retrieval solution (pH 9) at 125 °C for 30 min and then 90 °C for 10 min. The slides were blocked with peroxidase-blocking reagent (Dako) for 5 min and then with 10% bovine serum albumin for 30 min. The slides were further incubated with anti-claudin-4 antibody followed by horseradish peroxidase-labeled secondary antibody. The immunoreactive proteins were stained with DAB substrate (Dako). The slides were also stained with hematoxylin solution. Tissue sections were observed under a microscope.

4.3. Preparation of C-CPE-fused OVA

C-CPE-fused OVA (OVA-C-CPE) was prepared as described previously (Kakutani et al. 2010). Briefly, the plasmid pET-OVA-C-CPE was transduced into *Escherichia coli* BL21 (DE3), and the production of OVA-C-CPE was stimulated with isopropyl-D-thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol). The lysates were applied to HiTrap™ Chelating HP (GE Healthcare UK Ltd., Buckinghamshire, UK), and OVA-C-CPEs were eluted with buffer A containing 100-400 mM imidazole. The buffer was exchanged with phosphate-buffered saline (PBS) by using a PD-10 column (GE Healthcare UK Ltd.), and the purified protein was stored at −80 °C before use. Purification of the OVA-C-CPEs was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue. Protein was quantified by using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with BSA as a standard.

4.4. Nasal immunization and sample collection

Mice were nasally immunized once a week for 3 weeks at the indicated dose of OVA. For instance, a dose of 5 μg OVA is equal to a mixture of OVA (5 μg) and C-CPE (1.89 μg) or OVA-C-CPE (6.89 μg). Plasma was collected 7 days after the last nasal immunization.

4.5. OVA-specific IgG production

The titers of OVA-specific antibody in serum were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, an immunoplate was coated with OVA (100 μg/well in a 96-well plate). Ten-fold serial dilutions of these samples were added to the immunoplate followed by the addition of horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1 or IgG2a (Bethyl Laboratories Inc., Montgomery, TX). The OVA-specific antibodies were detected by using a TMB peroxide substrate (Thermo Fisher Scientific Inc., Rockford, IL). End-point titers were expressed as the reciprocal log of the last dilution ratio, which was 0.1 greater than the control values obtained for the serum of naïve mice at an absorbance of 450 nm.

4.6. Histological analysis of nasal mucosa in mice immunized with OVA-C-CPE

Mice from either the non-immunized group or the immunized group (6.89 μg of OVA-C-CPE) were sacrificed 7 days after immunization for histopathological analysis. Tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Sections (4 μm) were prepared for hematoxylin and eosin staining. Histopathologic examinations were performed at the Applied Medical Research Laboratory (Osaka, Japan). Inflammation was scored according to the severity of the inflammatory cell infiltration: 0, none; 1, weak; 2, moderate; and 3, severe. Scoring was performed blindly to avoid bias.

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References

Dhuria SV, Hanson LR, Frey WH (2010) Intranasal delivery to the central nervous system: mechanisms and experimental considerations. J Pharm Sci 99: 1654–1673.

Ebihara C, Kondoh M, Hasuike N, Harada M, Mizuguchi H, Horiguchi Y, Fujii M, Watanabe Y (2006) Preparation of a claudin-targeting molecule using a C-terminal fragment of *Clostridium perfringens* enterotoxin. J Pharmacol Exp Ther 316: 255–260.

Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, Noda T, Kubo A, Tsukita S (2002) Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 156: 1099–1111.

Furuse M, Tsukita S (2006) Claudins in occluding junctions of humans and flies. Trends Cell Biol 16: 181–188.

Ivanov AI, Nusrat A, Parkos CA (2004) Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. Mol Biol Cell 15: 176–188.

Kakutani H, Kondoh M, Fukasaka M, Suzuki H, Hamakubo T, Yagi K (2010) Mucosal vaccination using claudin-4-targeting. Biomaterials 20: 5463–5471.

Katahira J, Inoue N, Horiguchi Y, Matsuda M, Sugimoto N (1997) Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. J Cell Biol 136: 1239–1247.

Kondoh M, Masuyama A, Takahashi A, Asano N, Mizuguchi H, Koizumi N, Fujii M, Hayakawa T, Horiguchi Y, Watanbe Y (2005) A novel strategy for the enhancement of drug absorption using a claudin modulator. Mol Pharmacol 67: 749–756.

Kunisawa J, Nochi T, Kiyono H (2008) Immunological commonalities and distinctions between airway and digestive immunity. Trends Immunol 29: 505–513.

McClane BA (2001) *Clostridium perfringens*. In: Doyle MP, Beuchat LR, Montville TJ, editors. Food miclobiology: fundamentals and frontiers. Washington, DC: ASM Press: 352–372.

McClane BA, Chakrabarti G (2004) New insights into the cytotoxic mechanisms of *Clostridium perfringens* enterotoxin. Anaerobe 10: 107–114.

McClane BA, Lyerly DM, Moncrief JS, Wilkins TD (2000) Enterotoxic clostridia: *Clostridium perfringens* type A and *Clostridium difficile*. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, editors. Gram-positive pathogens. Washington, DC: ASM Press: 551–562.

Morita K, Furuse M, Fujimoto K, Tsukita S (1999) Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 96: 511–516.

Neutra MR, Kozlowski PA (2006) Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 6: 148–158.

Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, Tsukita S (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol 161: 653–660.

Rajapaksa TE, Stover-Hamer M, Fernandez X, Eckelhoefer HA, Lo DD (2010) Claudin 4-targeted protein incorporated into PLGA nanoparticles can mediate M cell targeted delivery. J Control Release 142: 196–205.

Saeki R, Kondoh M, Kakutani H, Tsunoda S, Mochizuki Y, Hamakubo T, Tsutsumi Y, Horiguchi Y, Yagi K (2009) A novel tumor-targeted therapy using a claudin-4-targeting molecule. Mol Pharmacol 76: 918–926.

Scheneeberger EE, Lynch RD (1992) Structure, function, and regulation of cellular tight junctions. Am J Physiol 262: L647–L661.

Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, Tsukita S (1999) *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier. J Cell Biol 147: 195–204.

Tamagawa H, Takahashi I, Furuse M, Yoshitake-Kitano Y, Tsukita S, Ito T, Matsuda H, Kiyono H (2003) Characteristics of claudin expression in follicle-associated epithelium of Peyer's patches: preferential localization of claudin-4 at the apex of the dome region. Lab Invest 83: 1045–1053.

Tsukita S, Furuse M, Itoh M (2001) Multifunctional strands in tight junctions. Nat Rev Mol Cell Biol 2: 285–293.

Uchida H, Kondoh M, Hanada T, Takahashi A, Hamakubo T, Yagi K (2010) A claudin-4 modulator enhances the mucosal absorption of a biologically active peptide. Biochem Pharmacol 79: 1437–1444.