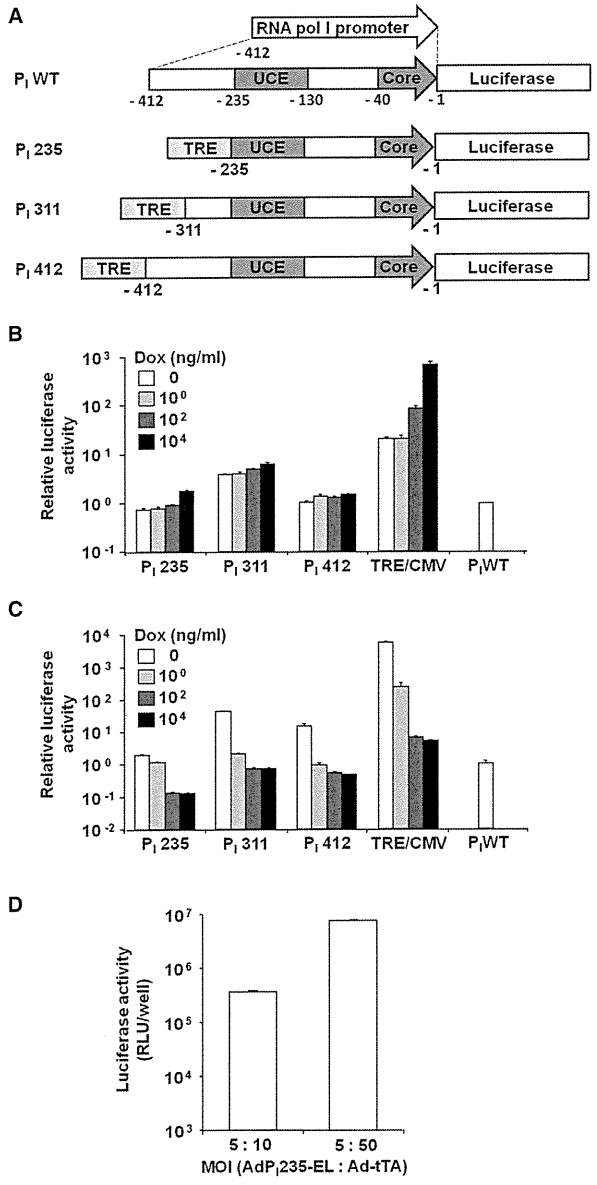

Figure 2. Development of tet-controllable RNA pol I promoter. (**A**) Construct of the chimeric RNA pol I promoter. The 412-bp human RNA pol I promoter contains Core (from −40 to −1), the binding site of the transcription factor, and UCE (from −235 to −130). TRE is connected to the full or partial RNA pol I promoter at the indicated sites, resulting in P_I235, P_I311 and P_I412. (**B** and **C**) Promoter activities of the chimeric promoter in Huh7 cells. Huh7 cells were co-transfected with the chimeric RNA pol I plasmid coding EGFPLuc, pCMVβ and *trans*-activator plasmid [rtTA (B) or tTA (C)]. After 2.5 h of transfection, the cells were treated with Dox at the indicated dose. After an additional 48 h of incubation, the luciferase and β-galactosidase activities were measured. The luciferase activity was normalized by the β-galactosidase activity and expressed relative to that of pP_IWT-EL-transfected cells. Data are mean ± SD (n = 3). (**D**) Transgene activity of Ad vector coding the chimeric promoter construct. Huh7 cells were co-infected with AdP_I235-EL and Ad-tTA. The MOI ratio of AdP_I235-EL to Ad-tTA was 5:10 or 5:50. After an additional 48 h of incubation, the luciferase activity was measured. Data are the mean ± SD (n = 3).

Luciferase was also expressed (Figure 3C). Dox dose-dependently attenuated expression of luciferase (Supplementary Figure S3). To discriminate between translation of the RNA pol I-transribed HCV RNA derived from the vector DNA and translation of HCV RNA derived from autonomous HCV replication in the transcribed cells, we prepared replication-incompetent HCV replicon deleting GDD motif in NS5B. Luciferase expression was attenuated in the cells transfected with the GDD-deleted Ad vector (AdP_I235-ΔGDD) (Figure 3D). A fragment of the HCV negative strand RNA, an essential replication intermediate, amplified by RT-PCR has been detected in the cells transfected with AdP_I235-HCV but not AdP_I235-ΔGDD (Figure 3E). Autonomous replication of the HCV RNA may occur in this system. To evaluate whether the Ad vector systems could be used to evaluate inhibitors of HCV replication, we investigated the effect of IFN on luciferase expression from HCV replicon in the Ad vector. As shown in Figure 3F, treatment of cells with 5 pg/ml of IFN reduced luciferase expression (33.3% of vehicle-treated cells). Cell viability was not affected by IFN treatment (Figure 3G). These findings indicate that the tet-controllable RNA pol I Ad vector may be useful for evaluation of anti-HCV activity.

DISCUSSION

HCV is an RNA virus containing the positive strand of a 9.6-kb RNA genome. A technique to transfer all or part of the HCV RNA genome to cells could be widely applicable for basic studies on HCV and pharmaceutical therapy against HCV. However, efficient and convenient methods to transduce the HCV RNA genome have never been fully developed. Electroporation of *in vitro* translated HCV RNA genome into cells is the most popular method. In the present study, we used a tet-controllable expression system to successfully develop an Ad vector system expressing the HCV RNA genome.

To our knowledge, development of Ad vector expressing HCV subgenome or genome has never been succeeded. The NS3 protease is essential for processing most of the NS proteins from the HCV polyprotein (27–30). The cleavage site of the NS3 protease is estimated to be between the P1 and P1′ position of an acidic amino acid at the P6 position, a Cys or Thr residue at the P1 position, and a Ser or Ala residue at the P1′ position (31). E1A, pIIIa, pol and V proteins of Ad have the cleavage site of the NS3 protease. The lack of previous success in generating Ad vectors coding the HCV genome and subgenome might be partly due to the degradation of Ad components by the NS3 protease during the preparation of Ad particles. In the tet-regulated system, when Ad vectors coding foreign genes driven by the TRE hybrid promoter are co-transfected with tTA or rtTA vector, the foreign gene can be expressed. Expression of the foreign gene could be suppressed during amplification of Ad vector particles in 293 cells, resulting in the preparation of Ad vector particles. The critical factor in the HCV replicon must be determined in a future study.

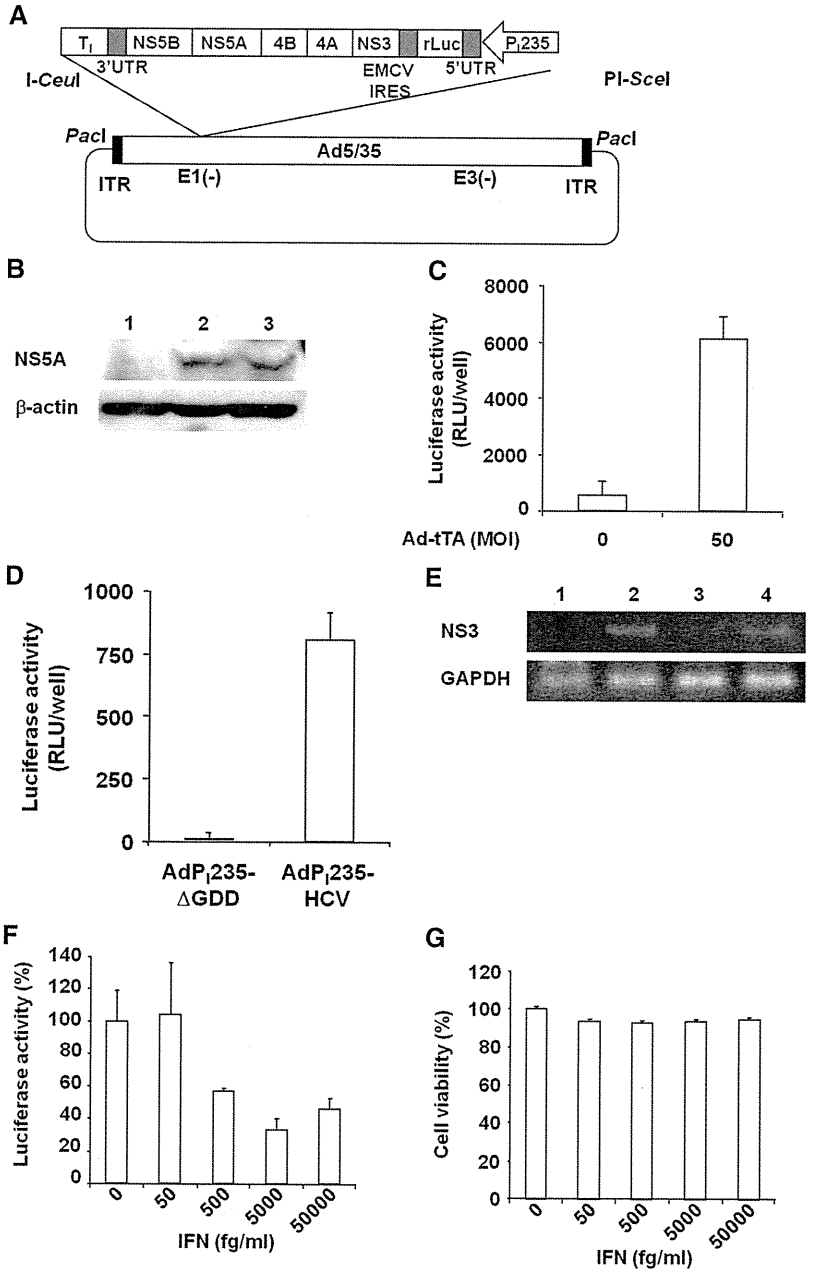

Figure 3. Preparation of Ad vector to monitor HCV replication. (**A**) Construct of Ad vector. The Ad vector contained the chimeric RNA pol I promoter (P_I235) and the HCV replicon to monitor HCV replication as the luciferase expression. (**B**) Expression of HCV NS5A protein in Huh7 cells transfected with AdP_I235-HCV. The cells were transfected with AdP_I235-HCV (10 MOI) and Ad-tTA (50 MOI). After 72 h of incubation, the cells were harvested, and the lysates (30 μg) were subjected to SDS–PAGE, followed by immunoblotting with antibody against NS5A. Huh7 cells and Huh7.5.1 1bFeo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7 cells infected with AdP_I235-HCV; lane 3, Huh7.5.1 1bFeo cells. (**C**) Expression of luciferase in the Ad vector-transfected cells. Huh7 cells were co-infected with AdP_I235-HCV (10 MOI) and 0 or 50 MOI of Ad-tTA. After an additional 48 h of incubation, the luciferase activity was measured. Data represent the mean ± SD ($n = 3$). (**D**) Involvement of NS5B in expression of luciferase in the Ad vector-transfected cells. Huh7 cells were infected with AdP_I235-HCV or AdP_I235-ΔGDD (3 MOI) and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then, the luciferase activity was measured. Data represent the mean ± SD ($n = 3$). (**E**) Expression of minus-stranded HCV RNA in the Ad vector-transfected cells. Huh7 cells were co-infected with AdP_I235-HCV or AdP_I235-ΔGDD at 3 MOI and Ad-tTA at 15 MOI. After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Then RT-PCR analysis was performed for detection of minus-stranded HCV NS3 and GAPDH. The PCR products were separated on 2% agarose gel. Huh7 cells and Huh7.5.1 1bFeo cells were used as the negative and positive controls, respectively. Lane 1, Huh7 cells; lane 2, Huh7.5.1 1bFeo cells; lane 3, Huh7 cells infected with AdP_I235-ΔGDD; lane 4, Huh7 cells infected with AdP_I235-HCV. (**F** and **G**) Effect of IFN on the replication of HCV replicon. Huh7 cells were infected with AdP_I235-HCV (10 MOI) and Ad-rtTA (50 MOI). After 1.5 h of infection, the cells were treated with IFN at the indicated concentration for 72 h. Then, the luciferase activity (F) and the cell viability (G) were measured. Data represent the percentage of vehicle-treated cells. Data are the mean ± SD ($n = 3$).

Transgenes delivered by a conventional Ad5 vector are limited to a size of 8.1–8.2-kb (32), and the size of HCV replicon is ~8.2-kb (containing a 1.0-kb luciferase gene and a 7.2-kb fragment of HCV genome) (8). The lack of a successful preparation of Ad5 vector may be partly due to limitation of packaging transgene. Mizuguchi and Hayakawa prepared a chimeric Ad vector containing type 5 and type 35 fiber proteins, which is a package 8.8-kb of foreign gene (26). CD46 is a receptor for Ad type 35 (Ad35), and CD46 is ubiquitously expressed in human cells (33,34). The Ad5/35 chimera vector can transduce various human cells more effectively than Ad5 vectors, indicating that the Ad5/35 vector may be a better system than Ad5 (26,35). In this study, we successfully prepared an Ad5/35 vector coding a tet-regulated RNA pol I-driven HCV replicon, and we found that the Ad5/35 vectors could be applied to evaluation of anti-HCV activity.

In conclusion, to the best of our knowledge, this is the first report to establish a novel strategy for the preparation of Ad vector expressing the HCV genome by using a tet-controllable expression system. Replication-incompetent HCV particles will be a promising candidate for vaccine therapy for HCV. As mentioned above, the packaging size (8.8-kb) of Ad5/35 vector used in the present study is smaller than that of the HCV RNA genome (9.6-kb), and, therefore, the preparation of inactive HCV particles using Ad5/35 vector is impossible. Helper-dependent Ad vector (HDAd), in which all viral coding sequences are deleted, can deliver a large capacity of ~37-kb to cells (36). Tet-controllable RNA pol I HDAd vector might contribute to the development of vaccine therapy for HCV.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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Mutated C-terminal fragments of *Clostridium perfringens* enterotoxin have increased affinity to claudin-4 and reversibly modulate tight junctions in vitro

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ABSTRACT

Passage across epithelial cell sheets is the first step in drug absorption. Tight junctions (TJs) are located between adjacent epithelial cells and seal the intercellular space preventing leakage of solutes. Claudin, a tetra-transmembrane protein family, is a pivotal functional and structural component of the TJ barrier. Modulation of the claudin-based TJ seal is a strategy for mucosal drug absorption. We previously found that a claudin-4 binder, a C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE194), was a modulator of the TJ seal and a potent mucosal absorption enhancer. In the present study, we attempted to improve claudin-4 binders by modification of C-CPE194. Substitution of Asn at position 309 and Ser at position 313 with Ala increased the affinity to claudin-4 by 9.9-fold as compared to C-CPE194. Deletion of 10 amino acids in the N-terminal domain of the double-alanine-substituted mutant increased affinity to claudin-4 by 23.9-fold as compared to C-CPE194. These C-CPE194 mutants reversibly modulated the TJ seal in human intestinal epithelial cell sheets. The N-terminal-truncated mutant was the most potent modulator of the TJ seal. These findings indicate that the C-CPE mutant may be a promising lead for the development of a clinical TJ modulator.

1. Introduction

Epithelium surrounds organisms and separates the inside of the body from the outside environment. Passage across the epithelium is the first step in drug absorption. Routes for solute movement across the epithelium are classified into transcellular and paracellular routes. Methods to deliver drugs through the transcellular route by simple diffusion or transporter- or receptor-mediated active transport have been developed since the 1990s, and some of these methods have been used clinically [1,2]. Absorption enhancers that deliver drugs through the paracellular route have been investigated since the 1960s, but most paracellular delivery systems have been investigated from the point of epithelial barrier-modulating activity [3,4]. Theoretical approaches for paracellular drug transport based on components of the epithelial barrier have never been fully developed because biochemical studies of the epithelial barrier have been sparse.

Tight junctions (TJs) are localized between adjacent epithelial cells and seal the intercellular space to prevent the leakage of solutes across the epithelial cell sheets. Modulation of the TJ barrier has been a strategy to enhance the epithelial absorption of drugs. However, biochemical and functional structures of TJs were not identified until 1998. Freeze-fracture replica electron microscopy analysis showed that TJs form a series of continuous, anastomotic and intramembranous particle strands, and the first structural and functional component of TJs, claudin, was identified in 1998 [5,6]. Claudin is a tetra-transmembrane protein with a molecular mass of ~23 kDa and comprises a family of 27 members [7,8]. Interestingly, the expression profiles and barrier functions of claudin family members differ among tissues. For instance, the epidermal barrier is disrupted in claudin-1-deficient mice, while the blood–brain barrier is deregulated in claudin-5-deficient mice [9,10]. These findings support the use of claudin-targeting strategies to enhance paracellular drug absorption.

Clostridium perfringens enterotoxin (CPE), a 35-kDa polypeptide, causes food poisoning in humans [11]. The CPE receptor is identical

Abbreviations: TJ, tight junction; C-CPE, the C-terminal fragment of *Clostridium perfringens* enterotoxin corresponding to 184-319 amino acids; CPE, *Clostridium perfringens* enterotoxin; DDM, *n*-dodecyl-β-D-maltoside; C-CPE194, C-terminal fragment of *Clostridium perfringens* enterotoxin corresponding to 194–319 amino acids; C-CPE205, C-terminal fragment of *Clostridium perfringens* enterotoxin corresponding to 205–319 amino acids; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BV, budded baculovirus; TBS, tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; TEER, transepithelial electric resistance.

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to claudin-3 and -4, and the C-terminal fragment of CPE (C-CPE; amino acids 184–319) binds to claudin-3/-4 [12–15]. Interestingly, C-CPE reversibly modulates TJ seals in epithelial cell sheets [15]. We previously found that the jejunal absorption-enhancing activity of a claudin binder was 400-fold more potent than a clinically used absorption enhancer, sodium caprate, and that the claudin binder enhanced jejunal, pulmonary and nasal absorption of a biologically active peptide [16,17]. Thus, claudin binders are promising absorption enhancers, but claudin binders have never been fully developed because of the difficulty in preparation of the claudin proteins needed for screening claudin binders and the low antigenicity of claudin. To develop claudin binders using C-CPE as a prototype, we previously prepared alanine-substituted mutants and N-terminus-truncated mutants, and we investigated their claudin-4-binding and TJ-modulating activities [17–19]. Based on these findings on the functional domain of C-CPE, we modified C-CPE and prepared claudin-4 binders with higher affinity and TJ-modulating activity in the present study.

2. Materials and methods

2.1. Reagents

n-Dodecyl-β-D-maltoside (DDM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Anti-claudin antibodies and anti-his-tag antibody were obtained from Invitrogen (Carlsbad, CA). CM5 sensor chips, amine-coupling reagents (*N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide, *N*-hydroxysuccinimide, and ethanolamine–HCl) and HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P20) were obtained from GE Healthcare (Buckinghamshire, UK). All reagents used were of research grade.

2.2. C-CPE mutants

C-CPE is the first claudin binder to be identified [15]. N-terminal region-truncated C-CPE mutants are also claudin-4 binders: C-CPE194 (amino acids 194–319) and C-CPE205 (amino acids 205–319) [17,20]. The DNA fragment encoding alanine-substituted C-CPE194 or C-CPE205 was amplified by polymerase chain reaction (PCR) using the alanine-substituted primers and a template plasmid encoding C-CPE194 or C-CPE205, respectively [17]. The resulting PCR products were cloned into a pET16 vector. The plasmids were transduced into *E. coli* BL21 (DE3), and protein expression was stimulated by the addition of isopropyl-1-thio-β-D-galactoside. C-CPE mutants were purified from the cell lysates by affinity chromatography with HisTrap™ HP (GE Healthcare). The solvent was exchanged with phosphate-buffered saline (PBS) by gel filtration, and the purified proteins were stored at −80 °C until used. Purification of the proteins was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining with Coomassie Brilliant Blue. C-CPE mutants were quantified using a BCA protein assay kit with bovine serum albumin as a standard (Thermo Fisher Scientific Inc., Rockford, IL).

2.3. Claudin-displaying budded baculovirus (BV)

Claudin-displaying BV was prepared as described previously [21]. Claudin-1 and -4 cDNA fragments were cloned into the baculoviral transfer vector pFastBac1 (Invitrogen). Recombinant baculoviruses were generated using the Bac-to-Bac system according to the manufacturer's instructions (Invitrogen). Sf9 cells were cultured in Grace's Insect medium (Invitrogen) containing 10% fetal bovine serum at 27 °C and infected with the recombinant baculovirus. Seventy-two hours after infection, the BV fraction was isolated from the culture supernatant of the infected Sf9 cells by centrifugation at 40,000*g* for 25 min. The pellets of the BV fraction were suspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail (Sigma–Aldrich, St. Louis) and then stored at 4 °C. The expression of claudins in the BV fraction was confirmed by SDS–PAGE and immunoblot with antibodies against claudins.

2.4. Enzyme-linked immunosorbent assay (ELISA)

The claudin-displaying BVs were adsorbed to the wells of 96-well immunoplates (Nunc, Roskilde, Denmark) overnight at 4 °C. The wells were blocked with 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) for 2 h at room temperature. C-CPE mutants were added to the wells and incubated for an additional 2 h at room temperature. The wells were incubated with anti-his-tag antibody for 2 h at room temperature. The immunoreactive proteins were detected by a horseradish peroxidase-labeled secondary antibody with 3,3′,5,5′-tetramethylbenzine as a substrate. The reaction was terminated by the addition of 0.5 M H_2SO_4, and the immunoreactive proteins were measured at 450 nm.

2.5. Recombinant claudin-4 protein

Recombinant claudin-4 protein was prepared by an expression system using Sf9 cells and recombinant baculovirus as previously reported [17]. Briefly, the C-terminal his-tagged claudin-4 cDNA fragment was cloned into pFastBac1, and recombinant baculovirus was generated using the Bac-to-Bac baculovirus expression system. Sf9 cells were infected with the recombinant baculovirus. After 52–56 h of infection, the cells were harvested by centrifugation. The cells were resuspended in a solution of 10 mM HEPES (pH 7.4), 120 mM NaCl with protease inhibitors (Complete Mini, EDTA-free, Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride and 20 U/ml DNase I. The cells were lysed with 2% DDM and then centrifuged. The resultant supernatant was applied to HisTrap™ HP, and claudin-4 was eluted with imidazole. The solvent for claudin-4 was changed to PBS containing 0.2% DDM by gel filtration with a HiTrap desalting column (GE Healthcare). Purification of claudin-4 was confirmed by SDS–PAGE followed by staining with Coomassie Brilliant Blue.

2.6. Surface plasmon resonance (SPR) analysis

SPR analysis was performed with a Biacore T100 instrument (GE Healthcare). Amine-coupling chemistry was used to immobilize claudin-4 at 25 °C on a CM5 sensor chip surface docked in a Biacore T100 and equilibrated with HBS-EP+. The carboxymethyl surface of the CM5 chip was activated for 2 min with a 1:1 ratio of 0.4 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide and 0.1 M *N*-hydroxysuccinimide at a flow rate of 10 μl/min. Claudin-4 was diluted to 2.5 μg/ml in 10 mM MES buffer (pH 6.5) and injected for 2 min over the surface at a flow rate of 10 μl/min. Excess activated groups were blocked by a 5-min injection of 1 M ethanolamine (pH 8.5) at a flow rate of 10 μl/min. Approximately 1000 RU of claudin-4 was immobilized using this protocol. Single-cycle kinetics experiments were performed at 25 °C with a flow rate of 30 μl/min [22]. C-CPE or its derivatives were serially diluted (1.25, 2.5, 5, 10 and 20 nM) in running buffer (HBS-EP+). Within a single binding cycle, samples of C-CPE or its derivatives were injected sequentially in order of increasing concentration over both the ligand and the reference surfaces. The reference surface, an unmodified flowcell, was used to correct for systematic noise and instrumental drift. Prior to the binding cycle for C-CPE or its derivatives, buffer was injected. These blank responses were used as a double-reference for the binding data [23]. The sensorgrams were globally fitted using a

1:1 binding model to determine k_a, k_d and K_D values with the Biacore T100 Evaluation Software version 2.0.1.

2.7. Transepithelial electric resistance (TEER) assay

Caco-2 cells were seeded onto Transwell™ chambers (Corning, NY) at a subconfluent density. The TEER of the Caco-2 monolayer cell sheets were monitored with a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). When TJs were developed reaching a plateau in the TEER values, the cells were treated with C-CPEs on the basal side of the chambers. Changes in TEER values were monitored. The TEER values were normalized by the area of the Caco-2 monolayer cell sheets, and the TEER value of a blank chamber was subtracted. Relative TEER values were calculated by the ratio to TEER in the vehicle-treated chambers or before treatment.

3. Results

3.1. Preparation of C-CPE mutants

C-CPE is a 400-fold more potent mucosal absorption-enhancer than a clinically used absorption-enhancer, sodium caprate [16]. Partial N-terminal-deleted C-CPE derivatives, C-CPE194 and C-CPE205, had over 30- and 10-fold higher solubility in PBS than C-CPE without loss of binding to claudin-4, respectively [17]. An alanine-substitution analysis of C-CPE indicated that replacement of Asn at position 309 or Ser at position 313 by alanine may improve its binding to claudin-4 and modulation of the TJ barrier [18]. Based on these findings, we speculated that the combination of N-terminal deletion and alanine substitution may produce a potent claudin modulator. We first focused on C-CPE194 because it has the highest solubility. We prepared C-CPE194$_{N309A}$, C-CPE194$_{S313A}$ and C-CPE194$_{N309A/S313A}$ (Fig. 1). To determine if these C-CPE mutants could bind to claudin-4, we performed ELISA with claudin-4-displaying BV. As shown in Fig. 2, all of these C-CPE mutants dose-dependently bound to claudin-4-displaying BV but not wild-type BV. Next, we quantitatively investigated their affinity to claudin-4 by SPR analysis (Table 1). Substitution of Asn at position 309 did not affect affinity to claudin-4 (K_D values of C-CPE194, 455 pM; C-CPE194$_{N309A}$, 451 pM). Replacement of Ser at position 313 by alanine elevated affinity to claudin-4 (K_D value of C-CPE194$_{S313A}$, 117 pM). Interestingly, the double alanine-substituted C-CPE194$_{N309A/S313A}$ had synergistically higher affinity to claudin-4 (K_D value, 46 pM). Based on these results, we tested the affinity of C-CPE205 with replacements of both the Asn at position 309 and the Ser at position 313 by alanine (C-CPE205$_{N309A/S313A}$) (Fig. 1). The affinity of C-CPE205$_{N309A/S313A}$ to claudin-4 was the highest (K_D value, 19 pM) among the C-CPE mutants (Table 1).

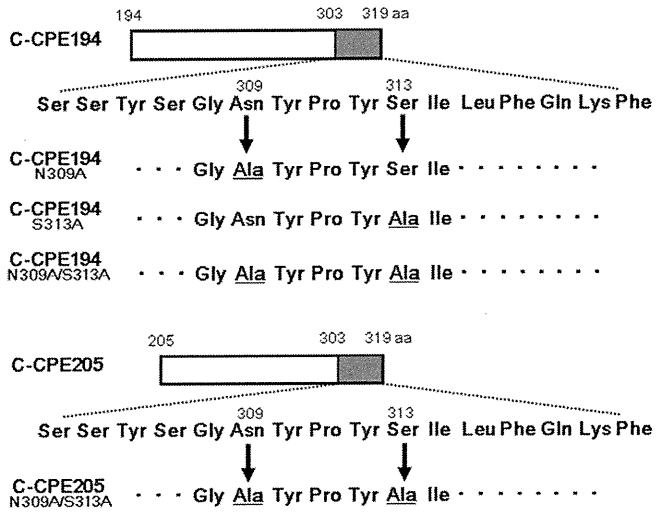

Fig. 1. Schematic illustration of C-CPE mutants. The C-terminal 16 amino acid fragment contains claudin-4-binding domains. A site-directed mutagenesis analysis of the C-terminal domain revealed that alanine substitution with Asn at position 309 or Ser at position 313 increased its binding to claudin-4 [18,19]. C-CPE194 and C-CPE205 are the C-terminal fragments of CPE corresponding to amino acids 194–319 and 205–319, respectively [17].

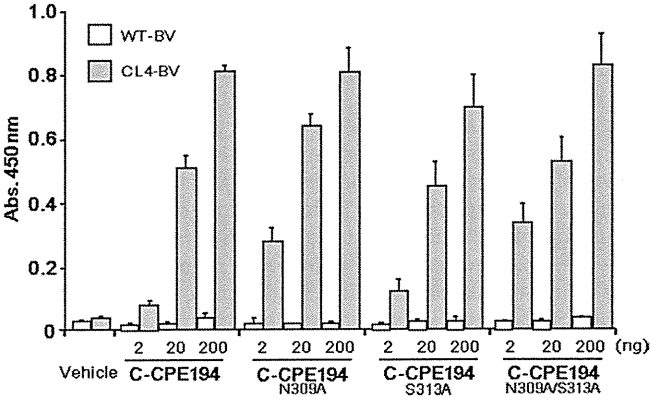

Fig. 2. Interaction of C-CPE mutants with claudin-4. Vehicle or C-CPE mutants were added to the wells of immunoplates coated with wild-type BV (WT-BV) or claudin-4-dysplaying BV (CL4-BV). After 2 h of incubation, anti-tag antibody, which recognized C-CPE mutants, was added to the wells, and then the C-CPE mutant-bound BV was detected by the addition of the horseradish peroxidase-labeled antibody, as described in Materials and methods. Data are representative of two independent experiments. The data are means ± SD ($n = 4$).

3.2. Effect of C-CPE mutants on the TJ seal

Next, we investigated the effects of C-CPE mutants on the modulation of the TJ seal. A monolayer culture of human colon Caco-2 cells is the most popular in vitro model for the assessment of TJ-seal modulation in the mucosal epithelium. As shown in Fig. 3A, treatment of monolayer cultures of Caco-2 cells with C-CPE mutants for 18 h dose-dependently decreased the integrity of TJ seals. The double alanine-substituted mutant C-CPE205$_{N309A/S313A}$ had the highest TJ-seal-modulating activity among the C-CPE mutants (CPE194: 62.7% and 31.7% of control at 5 and 20 μg/ml, respectively; C-CPE205$_{N309A/S313A}$: 14.6% and 10.3% of control at 5 and 20 μg/ml, respectively). The reversibility of TJ-seal modulation by a TJ modulator is important for safety. To investigate whether the C-CPE mutants reversibly modulated TJ integrity, C-CPE mutants were removed from the culture medium after 18 h, and then the cells were cultured for an additional 24 h. The TJ integrity was recovered 24 h after removal of the C-CPE mutants (Fig. 3B). These data indicate that the C-CPE mutants are reversible TJ modulators.

4. Discussion

Noninvasive drug administration is ideal for patient compliance and quality of life. Mucosal epithelium functions as a biological barrier preventing the free movement of solutes between the inside of the body and the outer environment. A strategy for noninvasive drug absorption is to modulate the TJ seal between adjacent mucosal epithelial cells. A C-terminal polypeptide fragment of CPE, referred to as C-CPE, modulates the TJ seal and binds to claudin-4 [15]. C-CPE enhanced the jejunal absorption of dextran by >400-fold as compared to a clinically used enhancer, and deletion of the claudin-4-binding domain attenuated its absorption-enhanc-

Table 1
Binding kinetics of C-CPE mutants to claudin-4.

Derivatives	*ka*(1/Ms)	*kd* (1/s)	K_D
C-CPE194	7.13×10^5	3.24×10^{-4}	455pM
C-CPE194$_{N309A}$	6.50×10^5	2.93×10^{-4}	451pM
C-CPE194$_{S313A}$	5.59×10^5	6.53×10^{-5}	117pM
C-CPE194$_{N309A/S313A}$	6.67×10^5	3.05×10^{-5}	46pM
C-CPE205$_{N309A/S313A}$	7.55×10^5	1.45×10^{-5}	19pM

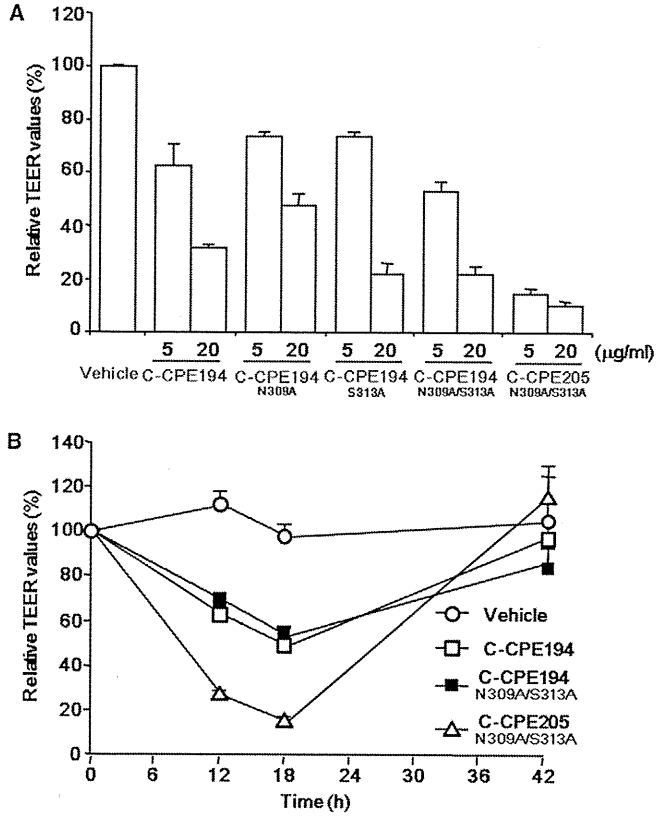

Fig. 3. Effect of C-CPE mutants on TJ-barrier in Caco-2 monolayer cell sheets. (A) Effect of C-CPE mutants on TJ-integrity. Caco-2 cells were cultured on Transwell™ inserts. When TEER values reached a plateau, the cells were treated with C-CPE or C-CPE mutants at 0, 5 or 20 μg/ml. After 18 h of C-CPE mutant treatment, the TEER values were monitored as described in the Materials and methods. The results were calculated as the percent of the TEER values to that of the vehicle-treated group. Data are representative of three independent experiments. The data are means ± SD ($n = 4$). (B) Reversibility of TJ modulation by C-CPE mutants. The cells were treated with C-CPE mutants (20 μg/ml) for 18 h, and then the cells were washed with the medium to remove the C-CPE mutants. The cells were cultured for an additional 24 h. Changes in TEER values were monitored during the C-CPE mutant treatment. The results were calculated as the percent of the TEER values to the values at 0 h. Data are representative of three independent experiments. The data are means ± SD ($n = 4$).

ing activity [16]. Claudin-4 binder may be useful for the development of a noninvasive drug delivery system. In the present study, we modified C-CPE based on our past functional domain mapping, and we found that the partial N-terminal-deleted and alanine-substituted mutants, C-CPE194$_{N309A/S313A}$ and C-CPE205$_{N309A/S313A}$, had higher affinity for claudin-4 and/or higher TJ-modulating activity than the parental C-CPE.

Why did substitution of Asn at position 309 and Ser at position 313 with Ala in C-CPE increase its affinity to claudin-4? Tyr residues at positions 306, 310 and 312 and Leu at position 315 in CPE are involved in the interaction between CPE and claudin-4 [18]. Winkler et al. proposed that C-CPE may interact with the hydrophobic turn in the second loop of claudin through its hydrophobic pit on the surface of C-CPE formed by those Tyr and Leu residues [24]. The substitution of Asn at position 309 and Ser at position 313 with Ala may reduce polarity and steric hindrance in the hydrophobic pit of C-CPE leading to increased binding of the Tyr and Leu residues to the hydrophobic region of claudin. Replacement of Ser at 305 or 307 by Ala also increased the binding of C-CPE to claudin-4 [18]. Tyr at position 306 is located between Ser residues at positions 305 and 307. Reduction of the polarity and steric hindrance surrounding Tyr at position 306, 310 and 312 and Leu at position 315 might increase the affinity of C-CPE to claudin-4.

C-CPE194$_{N309A/S313A}$ and C-CPE205$_{N309A/S313A}$ had 9.9- and 23.9-fold higher affinity to claudin-4 as compared to C-CPE194, respectively. However, their affinities to claudin-4 did not always affect their TJ-barrier-modulating activity. C-CPE205$_{N309A/S313A}$ modulated the TJ barrier more than C-CPE194, but C-CPE194$_{N309A/S313A}$ had TJ-barrier-modulating activity similar to C-CPE194. One possible explanation for this discrepancy may be differences in their cellular uptake activities. Claudin contains a clathrin-sorting signal in its C-terminal intracellular domain [25]. Treatment of cells with the C-terminal fragment of CPE caused a disappearance of claudin-4 in TJs and a decrease in claudin-4 protein [15]. Claudin-4 binders may first bind to claudin-4, after which the binders bound to claudin-4 may be taken up into the cytosol via endocytosis leading to the degradation of claudin-4. C-CPE205$_{N309A/S313A}$ might show higher endocytic activity by interacting with claudin-4 in a manner different than the other C-CPE mutants. These C-CPE mutants had similar association kinetics to claudin-4 but different dissociation kinetics to claudin-4 (Table 1). A longer interaction between C-CPE205$_{N309A/S313A}$ might increase the endocytosis of C-CPE mutant-bound claudin-4. Further experiments are needed to prove this hypothesis.

Preparation of antibodies against the extracellular domain of claudin-1, -3 and -4 has been recently reported [26–28]. However, to our knowledge, C-CPE is still the only modulator of the claudin barrier. C-CPE may be a promising lead for the development of TJ modulators. We have developed a screening system for claudin binders using a baculoviral display system [29]. We are attempting to develop novel claudin binders by the combination of a C-CPE mutant library and the baculoviral display system. Claudin-4-targeting is also a potent strategy for cancer-targeting and mucosal vaccination [21,27,30], for which C-CPE194$_{N309A/S313A}$ and C-CPE205$_{N309A/S313A}$ can be used. Taken together, our findings regarding C-CPE mutants will contribute to the development of not only drug-absorption enhancers but also claudin-targeted drug development, such as for cancer therapy and vaccines.

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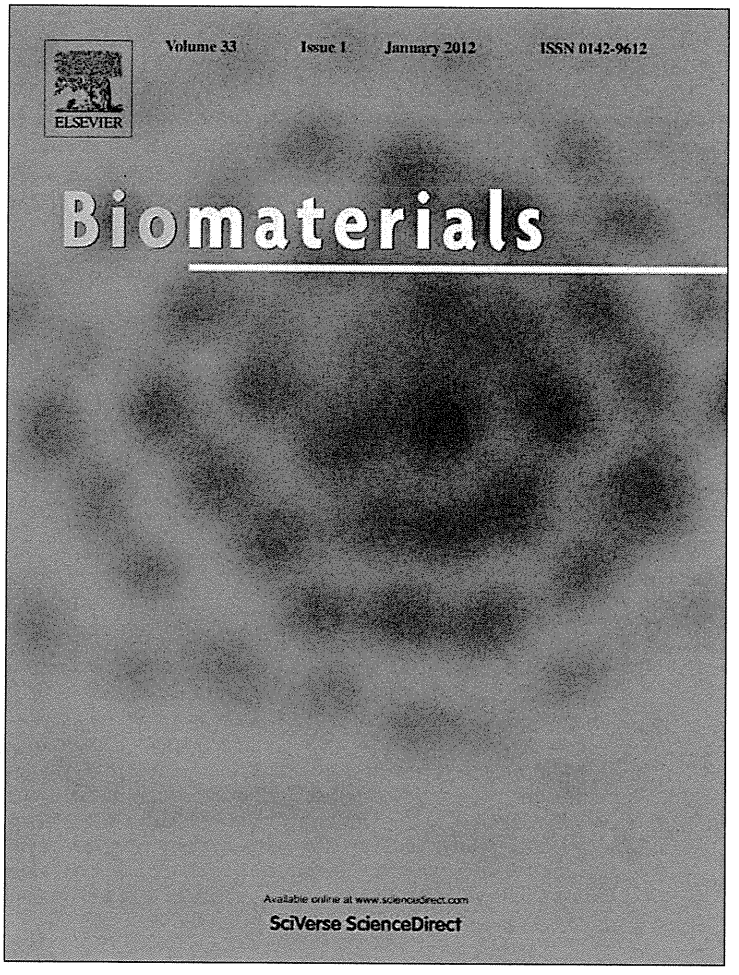

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The application of an alanine-substituted mutant of the C-terminal fragment of *Clostridium perfringens* enterotoxin as a mucosal vaccine in mice

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ABSTRACT

Efficient delivery of antigen to mucosal immune tissues is an essential part of mucosal vaccination. Claudin-4 is expressed on the epithelial cells that cover the mucosal immune tissues. We previously found that claudin-4-targeting is a promising strategy for mucosal vaccination by using a claudin-4 binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE). Substitution of Asn and Ser at positions 309 and 313, respectively, with alanine increased the affinity of C-CPE for claudin-4. However, application of the C-CPE mutant as a mucosal vaccine has never been tried. Here, we investigated whether the C-CPE mutant could serve as a mucosal vaccine. We used ovalbumin (OVA) as a model antigen and fused the C-CPE mutant to it. The resultant fusion protein was bound to claudin-4. When mice were immunized with the C-CPE mutant-fused OVA, OVA-specific serum IgG and nasal IgA increased relative to levels in mice immunized with a C-CPE-fused antigen. Immunization with the C-CPE mutant-fused OVA activated Th1- and Th2-type responses and led to increased anti-tumor activity against OVA-expressing thymoma cells relative to that of mice immunized with the C-CPE-fused antigen. These findings suggest that the alanine-substituted C-CPE mutant shows promise as a claudin-targeted mucosal vaccine.

1. Introduction

Vaccination is a potent therapeutic strategy for infectious diseases and cancers. Vaccines are classified as either parental or mucosal; parental vaccines are injected into patients, whereas mucosal vaccines are nasally, pulmonarily, or orally administered. Parental vaccines activate systemic immune responses but do not induce mucosal immunity. For parental vaccines, patient compliance is low and the preventive effects in response to invasion by pathological microorganisms into the body from the mucosal epithelium are poor. Mucosal vaccines, however, potentiate both systemic and mucosal immune responses. They eliminate invading pathogens and infected cells, and prevent the entry of pathogens from mucosal epithelium by producing antigen-specific IgA. Thus, mucosal vaccine is an ideal strategy for vaccination. However, mucosal administration of antigen alone does not activate immune responses; the delivery of antigens to the mucosal immune tissues is critical for the mucosal vaccine response.

Lymphoid immune tissues, called mucosa-associated lymphoid tissues (MALTs), reside in the mucosal epithelium, where they function as a first line of defense against pathogens invading the body via the epithelium by activating mucosal immune responses. MALTs comprise gut-associated lymphoid tissues (GALT), nasopharynx-associated lymphoid tissues (NALT), and bronchus-associated lymphoid tissues (BALT). MALTs contain lymphocytes, M cells, T cells, B cells, and antigen-presenting cells (APCs). Delivery of antigens to MALTs is essential for the activation of mucosal immune responses, and microparticles, liposomes, lectins, and chitosans have been used to deliver antigens to MALTs [1–5]. Follicle-associated epithelium

Abbreviations: C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin; OVA, ovalbumin; MALT, mucosa-associated lymphoid tissue; GALT, gut-associated lymphoid tissue; NALT, nasopharynx-associated lymphoid tissue; APC, antigen-presenting cell; FAE, follicle-associated epithelium; CPE, *Clostridium perfringens* enterotoxin; C-CPE184, C-terminal fragment of *Clostridium perfringens* enterotoxin from amino acids 184 to 319; C-CPE194, C-terminal fragment of *Clostridium perfringens* enterotoxin from amino acids 194 to 319; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; BV, budded baculovirus; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; IFN, interferon; IL, interleukin.

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(FAE) covers MALTs, and M cells in FAE are key antigen-sampling cells that present processed antigens to the APCs located below the M cells [6–8]. A ligand for FAE or M cells is a promising target molecule for mucosal vaccination. Claudin-4 was overexpressed in the FAE that covers GALTs and NALTs and in M cells [9–12]. These findings indicated that claudin-4-targeting is a promising strategy for mucosal vaccination.

Claudin, a tetra-transmembrane protein family with a molecular weight of ~23 kDa, comprises at least 27 family members [13,14]. Claudin-4 ligands show promise in the development of claudin-targeted mucosal vaccines; however, claudin-4 ligands, including antibodies, have never been fully developed because of low antigenicity and difficulty in preparing recombinant protein.

Clostridium perfringens enterotoxin (CPE) is a food poison in humans. Claudin-3 and -4 are receptors for CPE [15,16]. Interestingly, the C-terminal half of CPE binds to claudin-3 and -4 with no cytotoxicity [16,17]. This C-terminal fragment of CPE, which corresponds to amino acids 184–319 (C-CPE184), was the only known claudin binder prior to 2005. As such, it is the best characterized claudin binder. We previously found that C-CPE184 and the C-terminal fragment corresponding to amino acids 194–319, called C-CPE194, function as claudin-4-targeting ligands [18,19]. To investigate whether claudin-4-targeting could be a potent method for mucosal vaccination, we fused C-CPE184 with an experimental antigen and found that intranasal immunization of mice with the fusion protein activated antigen-specific mucosal and systemic immune responses and that these immune responses attenuated deletion of the claudin-4 binding domain. Thus, we demonstrated proof-of-concept that claudin-4 targeting may be a potent strategy for mucosal vaccination.

To develop a claudin binder with C-CPE184 as a prototype, we identified the functional residues of C-CPE184 by using serial deletion and site-directed mutagenesis. Substitution of certain residues with alanine increased binding to claudin-4, whereas deletion of the N-terminal 10 amino acids of C-CPE184 (C-CPE194) increased its solubility by over 30-fold over that of C-CPE184, with no change in affinity to claudin-4 [20–24]. Based on these findings, here, we prepared a fusion protein to serve as a model antigen containing a double alanine-substituted C-CPE194 mutant (Asn and Ser at position 309 and 313, respectively), and investigated mucosal vaccination by nasal immunization with the fused antigen.

2. Materials and methods

2.1. Preparation of C-CPEs and C-CPE-fused ovalbumin (OVA)

Fragments of alanine-substituted C-CPE194 were amplified by using polymerase chain reaction with C-CPE194 cDNAs as a template with appropriate primers for each mutant [23]. The resultant C-CPE fragments were cloned into the pET16b vector (Novagen, Darmstadt, Germany). Expression plasmids encoding fusion proteins of OVA with C-CPEs were prepared as follows: oligonucleotides containing the G4S linker and multi-cloning sites, including the KpnI, SpeI, SmaI, and PacI sites, were subcloned into NdeI-digested pET16b encoding C-CPEs, resulting in pET-MCS-C-CPEs. The OVA cDNA fragment was inserted into pET-MCS-C-CPEs at the KpnI/PacI site, resulting in pET-OVA-C-CPEs. The OVA-C-CPE-expression plasmids were transduced into *Escherichia coli* strain BL21 (DE3), and production of recombinant proteins was induced by the addition of 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™ HP (GE Healthcare, Buckinghamshire, UK), and the OVA-C-CPE proteins were eluted with imidazole. The solvent was exchanged with phosphate-buffered saline (PBS) by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80 °C until use. Purification of the recombinant proteins was confirmed by use of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by staining with coomassie brilliant blue. A protein assay was carried out by using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

2.2. Preparation of claudin-displaying budded baculovirus (BV)

Claudin-1 and -4 cDNAs were cloned into the baculoviral transfer vector pFastBac1 (Invitrogen, Gaithersburg, MD). Recombinant BVs were prepared by using the Bac-to-Bac system according to the manufacturer's protocols (Invitrogen). Briefly, Sf9 cells were infected with recombinant baculovirus. Seventy-two hours later, the BV fraction was isolated from the culture supernatant of the infected Sf9 cells by centrifugation. The pellets of the BV fraction were suspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis) and then stored at 4 °C. The claudin-displaying BVs were confirmed by use of SDS–PAGE and immunoblotting with anti-claudin antibodies.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The claudin-displaying BVs were adsorbed onto wells in immunoplates (Nunc, Roskilde, Denmark). After well blocking with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan), OVA-C-CPEs were added to the wells and incubated. The wells were then washed with PBS and incubated with an anti-his-tag antibody, followed by incubation with a horseradish peroxidase-labeled secondary antibody. The immune-reactive proteins were detected by use of the TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Inc., MD). Immuno-reactive proteins were measured at 450 nm.

2.4. Fluorescence activated cell sorting (FACS) analysis

L cells, claudin-1 or -4-expressing L cells (kindly provided by Dr. S. Tsukita, Kyoto University, Japan) were incubated with OVA-C-CPEs for 1 h at 4 °C, followed by incubation with an anti-his-tag antibody. Then, the cells were incubated with a fluorescein-labeled secondary antibody, and the OVA-C-CPE-bound cells were detected and analyzed with a flow cytometer (FACSCallibur, Becton Dickinson, NJ).

2.5. Animals

Female Balb/c mice and C57BL/6 mice (6–8-weeks-old) were purchased from SLC Inc. (Shizuoka, Japan). They were maintained in a room at 23 °C ± 1.5 °C with a 12-h light/dark cycle and were allowed free access to standard chow and water. After their arrival, the mice were maintained for at least 1 week to adapt to this environment before being used in the experiments. All animal experiments were performed according to the guidelines of Osaka University.

2.6. Nasal immunization

Balb/c mice were nasally immunized with OVA-C-CPEs once a week for three weeks at doses equivalent to 0.5, 1.0, or 5.0 μg of OVA. Seven days after the last immunization, serum and mucosal secretions (nasal washes, vaginal washes, and fecal extracts) were collected. Fecal pellets (100 mg) were suspended in 1 ml of PBS and extracted by vortexing for 10 min. The samples were centrifuged, and the resultant supernatants were used as fecal extracts. Nasal and vaginal mucosa were washed with 200 μl or 100 μl of PBS, respectively.

2.7. Production of an OVA-specific antibody

The titers of OVA-specific antibody in the serum, mucosal washes, and extracts were determined by using ELISA. Briefly, an immunoplate was coated with OVA (100 μg/well in a 96-well plate). Ten-fold serial dilutions of samples were added to the immunoplate and then reacted with horseradish peroxidase-conjugated anti-mouse IgG, IgG1, IgG2a, or IgA. The OVA-specific antibodies were detected by using the TMB peroxide substrate. End-point titers were expressed as the dilution ratio that gave 0.1 above the control values obtained from the serum of naïve mice at an absorbance of 450 nm.

2.8. Cytokine ELISA

Splenocytes were isolated from the spleens of immunized mice. After stimulation of the cells with vehicle or OVA, interferon-γ (IFN-γ) and interleukin-13 (IL-13) in the conditioned medium were measured with an ELISA kit according to the manufacturer's protocol (R&D Systems, Inc., MN).

2.9. Anti-tumor activity

C57BL/6 mice were nasally immunized with OVA-C-CPEs once a week for three weeks at a dose of 5 μg OVA. Seven days after the last immunization, a murine thymoma cell model EG7-OVA cells (1×10^6 cells) were subcutaneously inoculated into the mice. Tumor volumes were monitored and calculated by using the following equation: $a \times b \times b/2$, where "a" and "b" are the maximum and minimum diameters of the tumor, respectively.

3. Results

3.1. Preparation of OVA-C-CPEs

C-CPE184 was the only claudin-4 binder, and we previously proposed claudin-4-targeted mucosal absorption, cancer-targeting, and mucosal vaccination using C-CPE184 as a model for claudin-4 binding [9,16,18,25]. To improve the claudin-4-targeting system, we investigated the functional domain of C-CPE184 by preparing N-terminal and C-terminal truncated mutants, as well as alanine-substituted mutants. Substitution of Asn at position 309 or Ser at position 313 with Ala increased binding of C-CPE184 to claudin-4 [20]. Deletion of the N-terminal 10 amino acids in C-CPE184, to create C-CPE194 in this study, increased its solubility in PBS by 30-fold over that of C-CPE184 [24]. Given these results, we prepared double alanine-substituted C-CPE194 at positions 309 and 313 (C-CPE194$_{N309A/S313A}$) and found that C-CPE194$_{N309A/S313A}$ had higher affinity for claudin-4 than did C-CPE184 or C-CPE194 [22]. To investigate whether C-CPE194$_{N309A/S313A}$ could be used as a claudin-4-targeted mucosal vaccine, we prepared fusion proteins of the popular experimental antigen OVA with the C-CPEs (Fig. 1A–C). OVA-C-CPE303 served as a negative control, in which the claudin-4-binding domain was deleted [9,21].

To investigate the interaction of the OVA-C-CPEs with claudins, we performed an ELISA using claudin-displaying BV. OVA-C-CPE303 did not bind to wild-BV, claudin-1-displaying BV, or claudin-4-displaying BV (Fig. 2A). In contrast, OVA-C-CPE194 and OVA-C-CPE194$_{N309A/S313A}$ bound to claudin-4-displaying BV, but not to wild-BV or claudin-1-displaying BV. The double alanine-substituted mutant may, therefore, have a higher affinity than OVA-C-CPE194 for claudin-4 (Fig. 2A). To confirm the interaction of the OVA-C-CPEs with the claudins, we carried out FACS analysis with claudin-expressing cells. OVA-C-CPE194$_{N309A/S313A}$ specifically bound to claudin-4-expressing cells (Fig. 2B).

3.2. Mucosal vaccination using C-CPE mutants

Next, to evaluate mucosal vaccination with OVA-C-CPE194$_{N309A/S313A}$, mice were intranasally administered OVA-C-CPE194$_{N309A/S313A}$ once a week for three weeks. One week after the last immunization, we determined the serum OVA-specific IgG and OVA-specific IgA levels in nasal and vaginal washes, and fecal extracts. Administration of >0.5 μg of OVA-C-CPE194$_{N309A/S313A}$ markedly increased serum OVA-specific IgG compared to administration of OVA alone. Serum OVA-specific IgG levels were 11.2-fold higher in mice immunized with OVA-C-CPE194$_{N309A/S313A}$ than in mice immunized with OVA-C-CPE194 at a dose of 1.0 μg (Fig. 3A). Nasal, vaginal and fecal OVA-specific IgA levels were also increased in mice immunized with OVA-C-CPE194$_{N309A/S313A}$ 4.4-fold, 7.4-fold, >10-fold compared to those in mice immunized with OVA-C-CPE194, respectively (Fig. 3B–D).

Antigen-specific immune responses by mucosal vaccination are classified as Th1- and Th2-type responses [26,27]. Claudin-4-targeted antigen activates both Th1- and Th2-type immune responses [9]. To evaluate whether claudin-targeting using OVA-C-CPE194$_{N309A/S313A}$ also activates Th1- and Th2-type immune responses, we measured production of IgG1 (a Th2 response) and IgG2a (a Th1 response). OVA-specific IgG1 and IgG2a levels increased 165-fold and 27.0-fold,

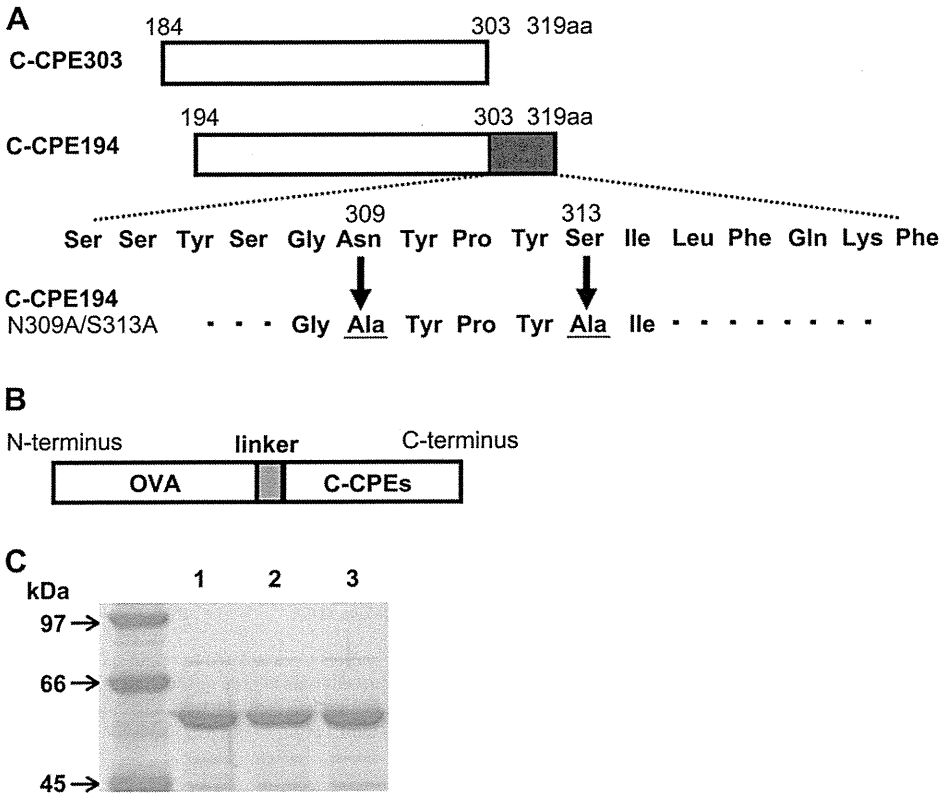

Fig. 1. Preparation of OVA-C-CPEs (A) Schematic illustration of C-CPEs. The C-terminal 16 amino acid-fragment contains the claudin-4 binding domains [21]; site-directed mutagenesis revealed that substitution of Asn at position 309 and Ser at position 313 with alanine increases claudin-4 binding [20]. C-CPE194 is the C-terminal fragment of CPE that corresponds to amino acids 194–319 [23]. (B) Schematic drawing of OVA-C-CPEs. OVA was linked with C-CPE303, C-CPE194, or C-CPE194$_{N309A/S313A}$ via a linker as described in the Materials and Methods. (C) Preparation of OVA-C-CPEs. OVA-C-CPEs were subjected to SDS–PAGE, followed by staining with coomassie brilliant blue. Lane 1, OVA-C-CPE303 (62 kDa); lane 2, OVA-C-CPE194 (62 kDa); lane 3, OVA-C-CPE194$_{N309A/S313A}$ (62 kDa). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

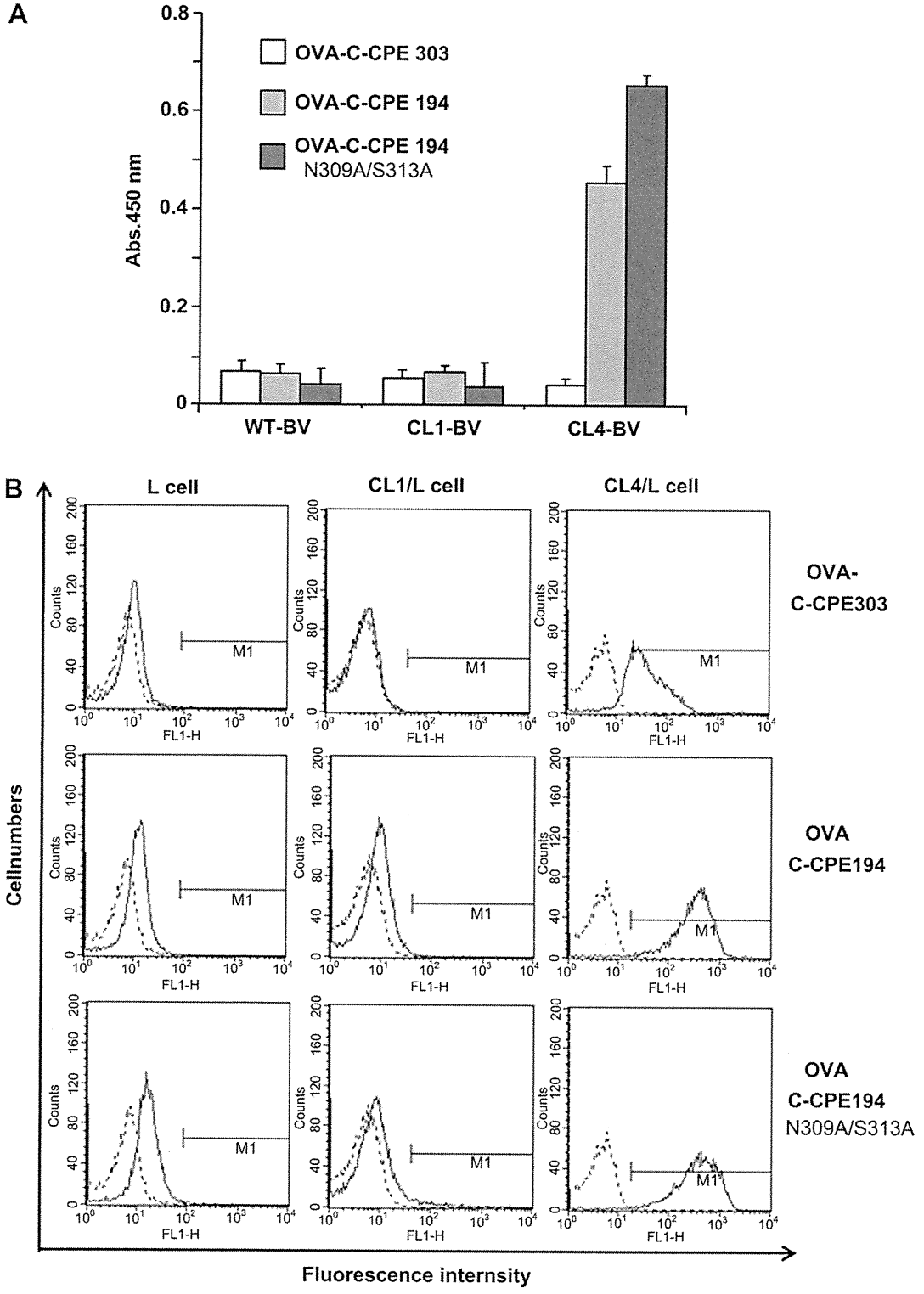

Fig. 2. Interaction of OVA-C-CPEs with claudin-4. (A) ELISA using claudin-displaying BV. An immunoplate was coated with wild-type BV (WT-BV), claudin-1-displaying BV (CL1-BV) or claudin-4-displaying BV (CL4-BV). Vehicle or OVA-C-CPE was added to each well. OVA-C-CPE-bound BV was detected by using an anti-his tag antibody and a peroxidase-labeled antibody as described in the Materials and Methods. Data are means ± SD ($n = 4$) and are representative of three independent experiments. (B) FACS analysis using claudin-expressing cells. L, claudin-1-expressing L cells (CL1/L cells) or claudin-4-expressing L cells (CL4/L cells) were treated with OVA-C-CPEs. Then, OVA-C-CPE-bound cells were detected by using an anti-his tag antibody followed by a fluorescein-labeled secondary antibody. Labeled cells were detected by use of FACS. The dotted line represents the histograms of the background in the absence of the primary antibody. The solid line indicates the histograms of the OVA-C-CPE-bound cells. Data are representative of three independent experiments.

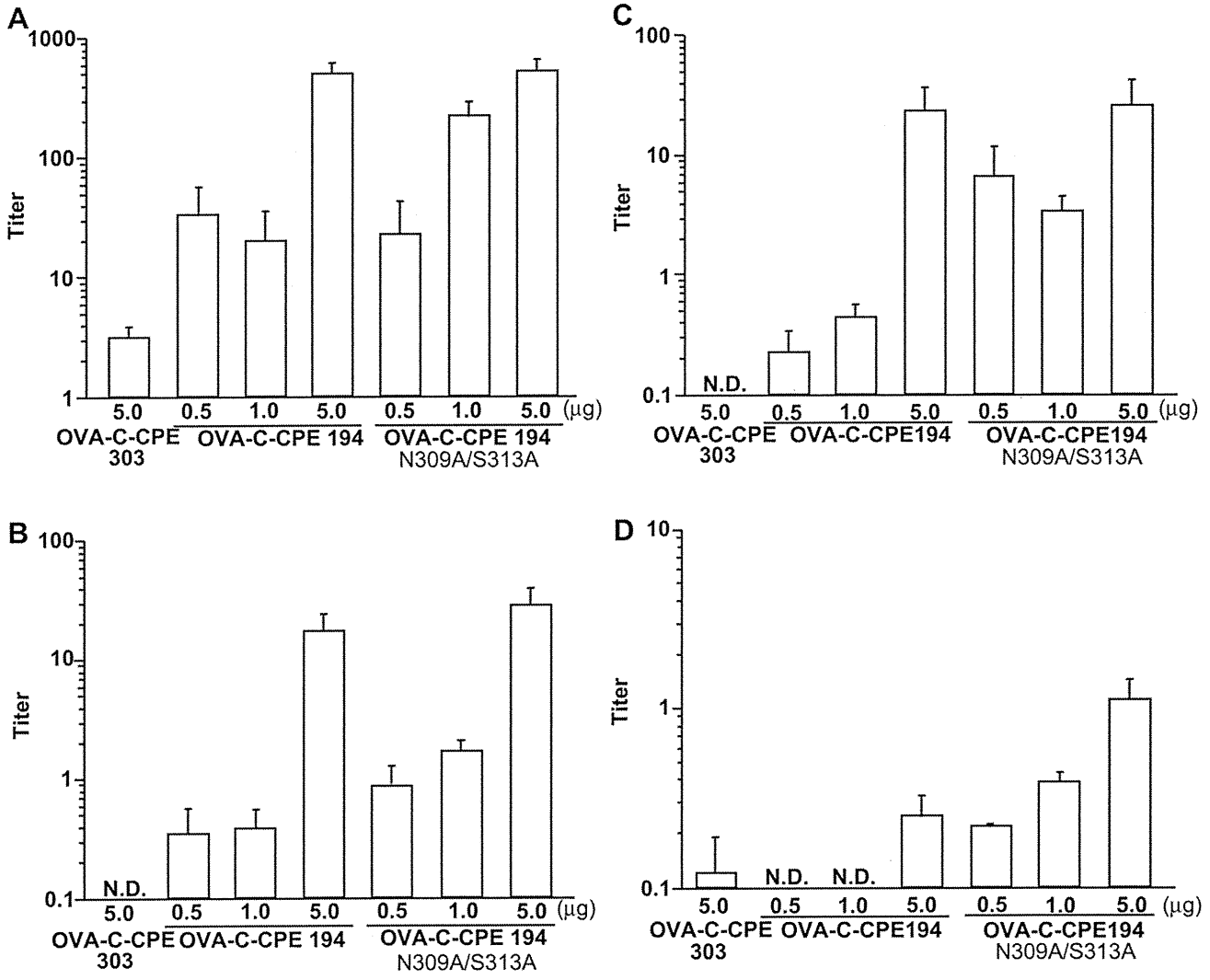

Fig. 3. Production of OVA-specific antibodies by nasal immunization with OVA-C-CPEs. Mice were nasally immunized with C-CPEs-fused OVA once a week for three weeks at the indicated doses. One week after the last immunization, serum, mucosal washes, and fecal extracts were recovered. OVA-specific serum IgG (A), nasal IgA (B), vaginal IgA (C) and fecal IgA (D) levels were measured as described in the Materials and Methods. Data are means ± SEM ($n = 4$).

respectively, in mice immunized with OVA-C-CPE194$_{N309A/S313A}$ compared to mice immunized with OVA-C-CPE194 (1 μg of OVA), respectively (Fig. 4A). Th1-specific (IFN-γ) and Th2- specific (IL-13) cytokines in splenocytes isolated from mice nasally immunized with OVA-C-CPE194$_{N309A/S313A}$ also increased 1.7-fold and 3.1-fold, respectively, relative to those from mice immunized with OVA-C-CPE194 (Fig. 4B).

Finally, we investigated the effect of mucosal vaccination on an experimental tumor model by using OVA-expressing cancer cells. Nasal immunization of mice with OVA or OVA-C-CPE303 did not suppress tumor growth (3044 ± 351 mm^3 in vehicle; 2250 ± 470 mm^3 in OVA; 3254 ± 573 mm^3 in OVA-C-CPE303 on day 24); however, immunization with OVA-C-CPE194 or OVA-C-CPE194$_{N309A/S313A}$ did suppress tumor growth (1143 ± 511 mm^3 and 399 ± 163 mm^3 on day 24, respectively). The double-alanine substituted mutant showed higher anti-tumor activity than that of OVA-C-CPE194 (Fig. 5). These results indicate that C-CPE194$_{N309A/S313A}$ may be a potent claudin ligand for mucosal vaccination.

4. Discussion

Efficient delivery of antigen to MALTs is pivotal for mucosal vaccination. Claudin-4 was expressed in FAE covering MALTs, and claudin-4-targeting has been proposed as a strategy for the development of mucosal vaccines [9,12]. The extracellular domain of claudin is weakly antigenic, and preparation of claudin proteins is difficult because of their hydrophobicity. Accordingly, the development of claudin-4 ligands has been limited. Here, we focused on C-CPE, which is the first identified claudin binder and found that the double alanine-substituted C-CPE194$_{N309A/S313A}$ can serve as a potent claudin ligand for the development of mucosal vaccination.

Efficient delivery of antigen to MALTs, which contain lymphocytes, M cells, T cells, B cells, and APCs, is an essential component of mucosal vaccine effectiveness [2]. Claudin-4 was expressed on FAE covering MALTs [9,12]. Lymphocytes, T cells, and B cells localize below the FAE [7,8]. FAE forms epithelial cell sheets that cover MALTs, and modulation of the TJ-seal in FAE can cause the influx of antigens to the immune-potent cells beneath the FAE. The claudin-4 binder disrupts the mucosal TJ-barrier [25]. C-CPE194$_{N309A/S313A}$ is a more potent modulator of the TJ-barrier than is C-CPE194 [22]. One possible explanation for the effectiveness of mucosal vaccination with C-CPE194$_{N309A/S313A}$ may be the influx of antigen into the immune-potent cells by modulation of the TJ-seal in the FAE. A mixture of C-CPE184 and OVA did not activate OVA-specific immune responses, whereas C-CPE184-fused OVA did. The targeting of antigen to claudin-4 is essential for mucosal vaccination

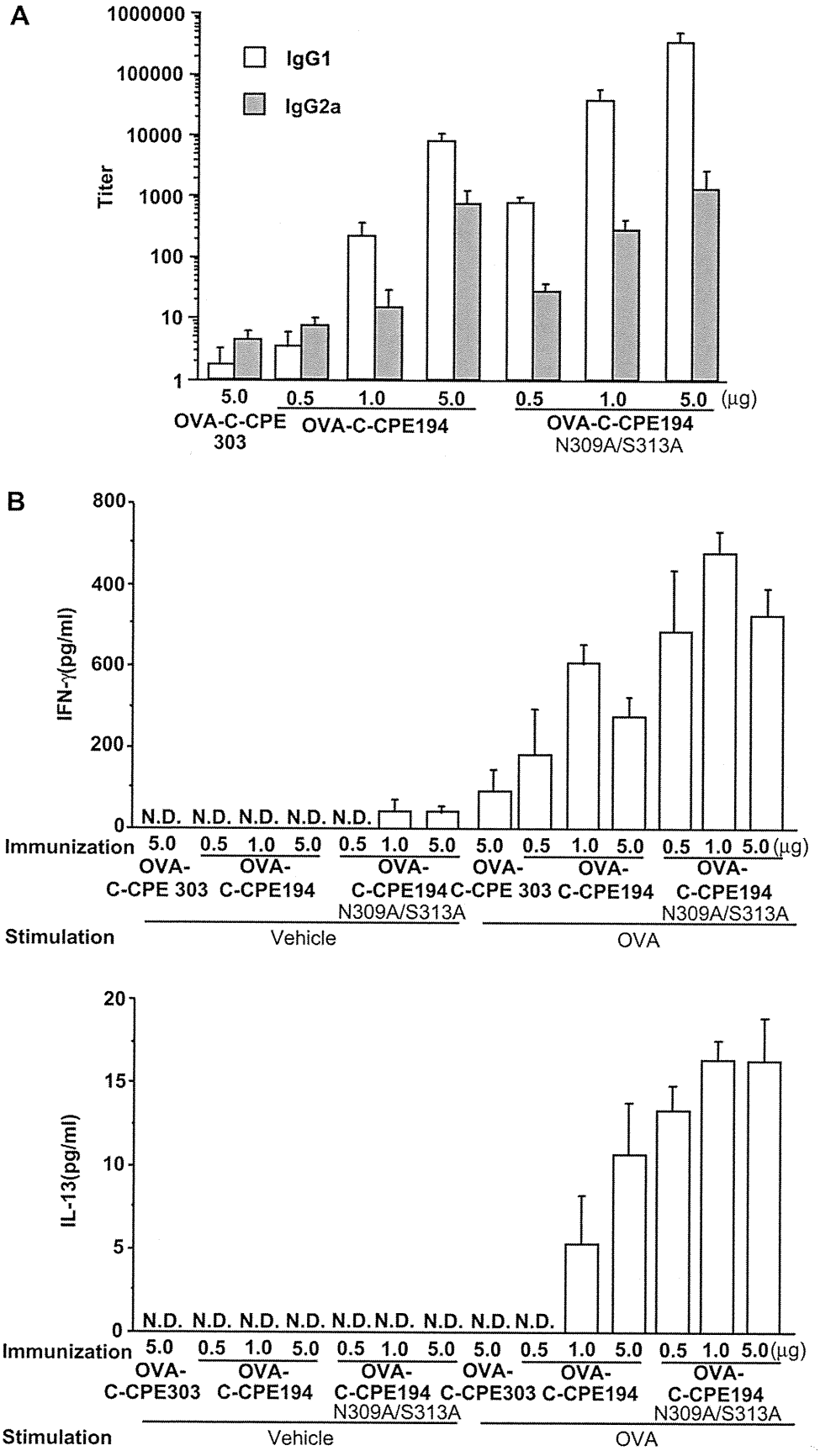

Fig. 4. Activation of Th1- and Th2-type immune responses by nasal immunization with OVA-C-CPEs. Mice were nasally immunized with C-CPEs-fused OVA once a week for three weeks at the indicated doses. One week after the last immunization, serum and splenocytes were recovered. OVA-specific serum IgG1 and IgG2a levels were measured as described in the Materials and Methods (A). The splenocytes were stimulated with OVA (1 mg/ml), and IFN-γ and IL-13 levels in the conditioned medium were measured by using a commercially available kit according to the manufacture's protocols (B). Data are means ± SEM ($n = 4$). The results are representative of three independent experiments.

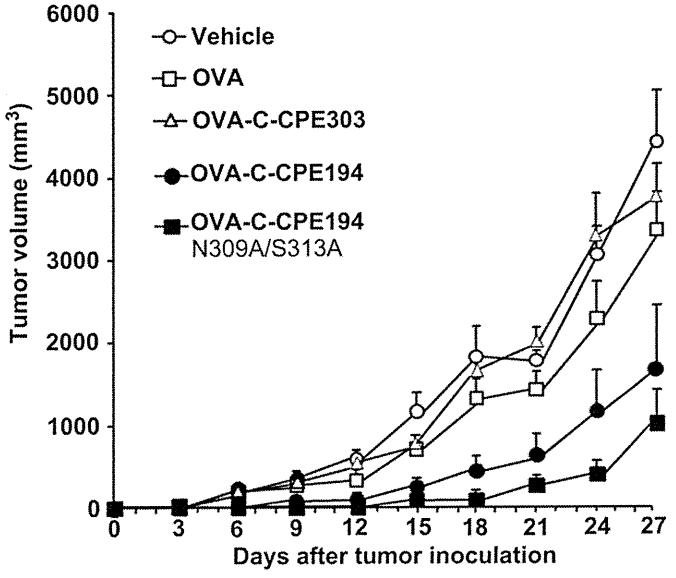

Fig. 5. Anti-tumor activity following immunization with OVA-C-CPE194$_{N309A/S313A}$. C57BL/6 mice were nasally immunized with vehicle, OVA, OVA-C-CPE303, OVA-C-CPE194, or OVA-C-CPE194$_{N309A/S313A}$ (5 μg of OVA) once a week for three weeks. Seven days after the last immunization, the mice were subcutaneously injected on the right back with 1×10^6 EG7-OVA cells. Tumor growth was monitored by measuring two diameters, and tumor volumes were calculated by using the following equation: $a \times b \times b/2$, where "a" is the maximum diameter of the tumor and "b" is the minimum diameter of the tumor. Data are means ± SEM ($n = 5$). The results are representative of two independent experiments.

using the claudin-4 binder [9], but the possibility of this may be negligible.

M cells are specialized antigen-sampling epithelial cells, beneath which various immune cells are localized. M cells deliver antigens from the lumen to the intraepithelial lymphoid cells via active transepithelial transport [6,28,29]. Rajapaksa et al. showed that claudin-4-targeted particles coated with the claudin-4-binding peptide are taken up by M cells, suggesting that claudin-4 is expressed on M cells [30]. Claudin-4 contains a clathrin-sorting signal in its intracellular C-terminal domain, and the claudin-4-targeting molecule is taken up by clathrin-mediated endocytosis [31]. The C-CPE194$_{N309A/S313A}$-fused antigen may be delivered more efficiently to M cells than the C-CPE194-fused antigen, followed by efficient delivery of antigen to the intraepithelial lymphoid cells. The association constant of C-CPE194$_{N309A/S313A}$ is 6.67×10^5 Ms^{-1}, which is similar to that of C-CPE194 (7.13×10^5 Ms^{-1}). In contrast, the dissociation constant of C-CPE194$_{N309A/S313A}$ is 3.05×10^{-5} S^{-1}, which is smaller than that of C-CPE194 (3.24×10^{-4} S^{-1}) [22]. C-CPE194$_{N309A/S313A}$-fused OVA might interact with claudin-4 longer than C-CPE194-fused OVA, resulting in efficient uptake of OVA into M cells. Claudin-4-targeting mucosal vaccination might be activated by uptake of antigen into M cells. Further analyses of the affinity of the fusion protein for claudin-4 and of the cellular uptake of the fusion protein are needed to clarify the mode of action.

Importantly, the claudin-4-targeting system activated immune responses without any adjuvant. Combining the claudin-4-targeting system with vaccine adjuvant may, therefore, create an even more effective mucosal vaccine. Antibodies are popular ligands for membrane proteins; however, the establishment of an anti-claudin antibody had been problematic because of its low antigenicity. Commercially available antibodies for claudin recognize the intracellular domain of claudins. Recently, two groups successfully prepared antibodies against the extracellular loop domain of claudin. Suzuki et al. immunized mice that had an autoimmune disease with claudin-4-expressing cells and raised an anti-claudin-4 antibody [32]. Fofana et al. immunized a rat with a DNA vector encoding claudin-1 to prepare an anti-claudin-1 antibody [33]. Soon, a claudin-targeted mucosal vaccine based on C-CPE mutants and/or antibodies will be developed.

5. Conclusions

We found that C-CPE194$_{N309A/S313A}$ worked as a potent claudin-4 ligand for the development of a mucosal vaccine. We could reduce the administered dose of the antigen using C-CPE194$_{N309A/S313A}$ more than C-CPE184 or C-CPE194, leading to reduction of the costs and the risk of anaphylaxis. Claudins have been considered as targets for mucosal vaccines, cancer therapy, and non-invasive drug absorption. The C-CPE mutant evaluated in this study shows promise as a lead claudin-4 ligand for claudin-targeted drug development.

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Use of human hepatocyte-like cells derived from induced pluripotent stem cells as a model for hepatocytes in hepatitis C virus infection

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ABSTRACT

Host tropism of hepatitis C virus (HCV) is limited to human and chimpanzee. HCV infection has never been fully understood because there are few conventional models for HCV infection. Human induced pluripotent stem cell-derived hepatocyte-like (iPS-Hep) cells have been expected to use for drug discovery to predict therapeutic activities and side effects of compounds during the drug discovery process. However, the suitability of iPS-Hep cells as an experimental model for HCV research is not known. Here, we investigated the entry and genomic replication of HCV in iPS-Hep cells by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively. We showed that iPS-Hep cells, but not iPS cells, were susceptible to infection with HCVpv. The iPS-Hep cells expressed HCV receptors, including CD81, scavenger receptor class B type I (SR-BI), claudin-1, and occludin; in contrast, the iPS cells showed no expression of SR-BI or claudin-1. HCV RNA genome replication occurred in the iPS-Hep cells. Anti-CD81 antibody, an inhibitor of HCV entry, and interferon, an inhibitor of HCV genomic replication, dose-dependently attenuated HCVpv entry and HCV subgenomic replication in iPS-Hep cells, respectively. These findings suggest that iPS-Hep cells are an appropriate model for HCV infection.

1. Introduction

Hepatitis C virus (HCV), a hepatotropic member of the *Flaviviridae* family, is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. Approximately 130–200 million people are estimated to be infected with HCV worldwide. Each year, 3–4 million people are newly infected with HCV [1]. Thus, overcoming HCV is a critical issue for the World Health Organization.

HCV contains a positive strand ~9.6 kb RNA encoding a single polyprotein (~3000 aa), which is cleaved by host and viral proteases to form structural proteins (core, E1, E2, and p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [1]. These virus proteins might be potent targets for anti-HCV drugs. However, combination therapy with interferon and ribavirin, which often causes severe side-effects leading to treatment termination, has been the only therapeutic choice [2]. Very recently, new direct antiviral agents have been approved or are under clinical trials; these agents include NS3 protease inhibitors, NS5A inhibitors, and NS5B polymerase inhibitors [2–4]. However, the emergence of drug resistance is a serious problem associated with the use of direct antiviral agents [5].

Host targets are alternative targets for the development of anti-HCV drugs. A liver-specific microRNA (miRNA), miR-122, facilitates the replication of the HCV RNA genome in cultured liver cells [6]. Administration of a chemically modified oligonucleotide complementary to miR-122 results in long-lasting suppression of HCV with no appearance of resistant HCV in chimpanzees [7]. Epidermal

Abbreviations: HCV, hepatitis C virus; iPS-Hep cells, human induced pluripotent stem cells-derived hepatocyte-like cells; HCVpv, HCV pseudotype virus; SR-BI, scavenger receptor class B type I; miRNA, microRNA; EGF-R, epidermal growth factor receptor; EphA2, ephrin factor A2; iPS cells, human induced pluripotent stem cells; FCS, fetal calf serum; Ad, adenovirus; HNF-4α, hepatocyte nuclear factor-4α; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VSV, vesicular stomatitis virus; VSVpv, VSV pseudotype virus; tet, tetracycline; pol, polymerase; MOI, multiplicity of infection; Dox, doxycycline; IFN, interferon-α8; ES cells, embryonic stem cells.

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growth factor receptor (EGF-R) and ephrin factor A2 (EphA2) are host cofactors for HCV entry [8]. Inhibitors of EGF-R and EphA2 attenuated HCV entry, and prevented the appearance of viral escape variants [8]. These findings strongly indicate that identification of host factors associated with infection of human liver by HCV is a potent strategy for anti-HCV drug development. Because the host tropism of HCV is limited to human and chimpanzee [9], there is no convenient model for the evaluation of HCV infections. This has led to a delay in the development of anti-HCV agents targeting host factors.

Takahashi and Yamanaka developed human induced pluripotent stem (iPS) cells from human somatic cells [10]. The stem cells can be redifferentiated *in vitro*, leading to new models for drug discovery, including iPS-based models for drug discovery, toxicity assessment, and disease modeling [11,12].

Recently, several groups reported that iPS cells can be successfully differentiated into hepatocyte-like (iPS-Hep) cells that show many functions associated with mature hepatocytes [13–19]. However, whether iPS-Hep cells are suitable as a model for HCV infection has not been fully determined. Here, we investigated HCV entry and genomic replication in iPS-Hep cells by using HCV pseudotype virus (HCVpv) and HCV subgenomic replicons, respectively.

2. Materials and methods

2.1. Cell culture

Huh7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). An iPS cell-line (Dotcom) generated from the human embryonic lung fibroblast cell-line MCR5 was obtained from the Japanese Collection of Research Bioresources Cell Bank [20,21]. The iPS cells were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore, Billerica, MA) in iPSellon culture medium (Cardio, Hyogo, Japan) supplemented with 10 ng/ml fibroblast growth factor-2.

2.2. In vitro differentiation

Before the initiation of cellular differentiation, the medium of the iPS cells was replaced with a defined serum-free medium, hESF9, and the cells were cultured as previously reported [22]. The iPS cells were differentiated into iPS-Hep cells by using adenovirus (Ad) vectors expressing SOX17, the homeotic gene HEX or hepatocyte nuclear factor 4α (HNF-4α) in addition to the appropriate growth factors, cytokines, and supplements, as described previously [19].

2.3. Reverse transcription (RT)-polymerase chain reaction (PCR) analysis of HCV receptors

Total RNA samples were reverse-transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), and the resultant cDNAs were PCR amplified by using Ex Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) and specific paired-primers for CD81 (5′-cgccaaggatgtgaagcagttc-3′ and 5′-tcccggagaagaggtcatcgat-3′), scavenger receptor class B type I (SR-BI; 5′-attccgatcagtgcaa catga-3′ and 5′-cagttttgcttcctgcagcacag-3′), claudin-1 (5′-tcagcact gccctgccccagt-3′ and 5′-tggtgttgggtaagaggttgt-3′), occludin (5′-tca gggaatatccacctatcacttcag-3′ and 5′-catcagcagcagccatgtactcttcac-3′), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5′-tct tcaccaccatggagaag-3′ and 5′-accacctggtgctcagtgta-3′). The expected sizes of the PCR products were 245 bp for CD81, 788 bp for SR-BI, 521 bp for claudin-1, 189 bp for occludin, and 544 bp for GAPDH. The PCR products were separated on 2% agarose gels and visualized by staining with ethidium bromide.

2.4. HCVpv infection

Pseudotype vesicular stomatitis virus (VSV) bearing HCV envelope glycoproteins (HCVpv) and VSV envelope glycoproteins (VSVpv) were prepared as described previously [23]. iPS, iPS-Hep and Huh7 cells were treated with HCVpv or mixtures of HCVpv or VSVpv and anti-CD81 monoclonal antibody (JS-81; BD Biosciences, Franklin Lakes, NJ) or control mouse IgG for 2 h. After an additional 24 h of culture, the luciferase activities were measured by using a commercially available kit (PicaGene, Toyo Ink, Tokyo, Japan).

2.5. Preparation of Ad vector expressing the HCV replicon

Ad vectors expressing a tetracycline (tet)-controllable and RNA polymerase (pol) I promoter-driven HCV subgenomic replicon containing renilla luciferase (AdP$_i$235-HCV), a replication-incompetent HCV subgenomic replicon containing renilla luciferase (AdP$_i$235-ΔGDD), tet-responsive trans-activator (Ad-tTA) or a tet-controllable RNA pol-I driven firefly luciferase (AdP$_i$235-fluc) were prepared by using an *in vitro* ligation method as described previously [24–26]. The biological activity (infectious unit) of the Ad vectors was measured by using an Adeno-X rapid titer kit (Clontech, Mountain View, CA).

2.6. HCV replication assay

iPS, iPS-Hep and Huh7 cells were infected with AdP$_i$235-HCV or AdP$_i$235-ΔGDD at multiplicity of infection (MOI; infectious unit per cell) of 3, and Ad-tTA at MOI of 15. After 24 h, the cells were treated with 10 μg/ml of doxycycline (Dox) for 48 h. Renilla luciferase activities in the lysates were then measured with the use of the Renilla Luciferase Assay System (Promega, Madison, WI). To normalize for the infectivity of Ad vector, iPS, iPS-Hep and Huh7 cells were co-infected with AdP$_i$235-fluc (3 MOI) and Ad-tTA (15 MOI). After a 72-h incubation, the firefly luciferase activities in the lysates were measured, and the renilla luciferase activities were normalized by dividing by the corresponding firefly luciferase activities.

2.7. Quantitative analysis of plus- and minus-strand HCV RNA

iPS, iPS-Hep and Huh7 cells were co-infected with AdP$_i$235-HCV or AdP$_i$235-ΔGDD (3 MOI), and Ad-tTA (15 MOI). After 24 h, the cells were treated with 10 μg/ml of Dox for 48 h. Total RNA was reverse-transcribed into cDNA by using the Thermoscript reverse transcriptase kit (Invitrogen) as described previously [27,28]. Real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa Bio Inc.) by using Applied Biosystems StepOne Plus (Applied Biosystems, Foster City, CA). The transcription products of the HCV plus-strand RNA, minus-strand RNA, and GAPDH gene, were amplified by using specific primers for HCV plus-strand RNA (RC1 primer, 5′-gtctagc-catggcgttagta-3′; and RC21 primer, 5′-ctcccggggcactcgcaagc-3′), HCV minus-strand RNA (tag primer, 5′-ggccgtcatggtggcgaataa-3′; and RC21 primer), and GAPDH (5′-ggtggtctcctctgacttcaaca-3′ and 5′-gtggtcgttgagggcaatg-3′), respectively. The copy numbers of the transcription products of the HCV plus- and minus-strand RNA were normalized with those of the GAPDH gene and infectivity of Ad vector as described in the Section 2.6.

2.8. Inhibition of HCV replication by interferon-α8

iPS-Hep and Huh7 cells were infected with AdP$_i$235-HCV (3 MOI) and Ad-tTA (15 MOI). After 24 h of infection, the cells were treated with 10 μg/ml of Dox and recombinant human interferon-α8 (IFN) at the indicated concentration. After an additional 48-h incubation, renilla luciferase activity in the lysates was measured with the use of the Renilla Luciferase Assay System. Cell