

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 measurements were 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 EDC and 0.1 M NHS at a flow rate of 10  $\mu$ l/min. Claudin-4 was diluted to 2.5  $\mu$ g/ml in 10 mM MES buffer (pH 6.5) and injected for 2 min over the surface at a flow rate of 10  $\mu$ l/min. Excess activated groups were blocked by a 5-min injection of 1 M ethanolamine (pH 8.5) at a flow rate of 10  $\mu$ l/min. Approximately 1000 RU of claudin-4 was immobilized by using this protocol. Single-cycle kinetics experiments were performed at 25 °C with a flow rate of 30  $\mu$ l/min [27]. 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. Also, 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 [28]. The sensorgrams were globally fitted by 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 BD BioCoat™ Fibrillar Collagen Cell culture inserts (BD Biosciences, San Jose, CA) at a density of  $1 \times 10^5$  cells/insert and cultured for 5 days. TJ barriers were formed by a 3-day culture in Entero-STIM™ (BD Biosciences) medium for cellular differentiation. C-CPE or its derivatives were added to the apical side of the chamber. After 18 h of incubation, the TEER values were measured with a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). The percentage changes of TEER values were calculated by the ratio to TEER value in 100  $\mu$ g/ml of C-CPE184. EC50 values, at which the TEER ratio is 50%, were calculated by using the four-parameter logistic function of DeltaSoft version 3 (BioMetallics, Princeton, NJ) from dose-response curves of the TEER ratio.

## 2.8. In situ loop assay

Jejunal absorption of hPTH(1-34) or fluorescein isothiocyanate-dextran with a molecular mass of 4 kDa (FD-4) was evaluated by using an in situ loop assay as described previously [21]. The experiments were performed according to the guidelines of the ethics committee of Osaka University or Asubio Pharma Co. Ltd. After 7-week-old Wister male rats were anesthetized with pentobarbital, a midline abdominal incision was made, and the jejunum was washed with PBS. A 5-cm long jejunal loop was prepared by closing both ends with sutures. hPTH(1-34) (100  $\mu$ g) was co-administered with C-CPEs into the loop or administered 4 h after the administration of C-CPEs. Blood was collected from the femoral artery by using a cannulated polyethylene tube at the indicated time points. EDTA (1 mg/ml) was immediately added to the blood sample, and the plasma was recovered by centrifugation. To avoid degradation of hPTH(1-34), aprotinin (500 IU/ml) was immediately added to the plasma, and the plasma was stored at -80 °C until use. The plasma hPTH(1-34) was quantified by

radioimmunoassay (RIA) with anti-hPTH antibody. Anti-hPTH antibody was added to the plasma and then incubated with [<sup>125</sup>I-Tyr34] hPTH(1-34) (15,000–20,000 cpm/100  $\mu$ l) for 24 h. The anti-rabbit IgG goat antibody was added, and anti-hPTH antibody bound to the anti-rabbit IgG goat antibody was separated by centrifugation. The radioactivity in the sediment was counted with a gamma counter (PerkinElmer Inc., Waltham, MA). The area under the plasma concentration time curve (AUC) from 0 to 120 min after administration was calculated by the trapezoidal method. Relative bioavailability (BA) was calculated with the following equation: BA (%) = (AUC (ng·min/ml)/dose ( $\mu$ g/kg))/(AUC (iv)(ng·min/ml)/dose (iv) ( $\mu$ g/kg)). AUC (iv) indicates the AUC<sub>0–120 min</sub> of intravenously administered hPTH(1-34) (10  $\mu$ g/kg), and the AUC value is  $208.6 \pm 52.7$  ng·min/ml.

Rats were anesthetized with thiamylal sodium, and a jejunal loop was made, as described above. A mixture of FD-4 (2 mg) and C-CPEs was co-administered into the jejunal loop. Blood was collected from the jugular vein at the indicated time points. The plasma levels of FD-4 were measured with a fluorescence spectrophotometer (Fluoroskan Ascent FL; ThermoElectron Corporation, Waltham, MA). The AUC of FD-4 from 0 to 6 h (AUC<sub>0–6 h</sub>) was calculated by the trapezoidal method.

## 2.9. Nasal and pulmonary absorption assay

Nasal and pulmonary absorption of hPTH(1-34) was examined in 7-week-old Sprague–Dawley male rats. The experiments were performed according to the guidelines of the ethics committee of Asubio Pharma Co. Ltd. For the nasal absorption assay, 200  $\mu$ g of hPTH(1-34) was intranasally administered to both sides of the nasal cavity 0 or 4 h after nasal administration of C-CPEs. The total injection volume did not exceed 20  $\mu$ l. For the pulmonary absorption assay, a polyethylene tube (PE-240, Clay Adams, Becton Dickinson & Co., Sparks, MD) was inserted into the trachea of each rat. A MicroSprayer (Penn-Century, Inc., Philadelphia, PA) was used to perform pulmonary injections of C-CPEs; then, after 0 or 4 h, 150  $\mu$ g of hPTH(1-34) was administered with the MicroSprayer. Blood was collected at the indicated time points, and the plasma concentration of hPTH(1-34) was measured by RIA, as described above. AUC and BA (%) values were calculated, as described above.

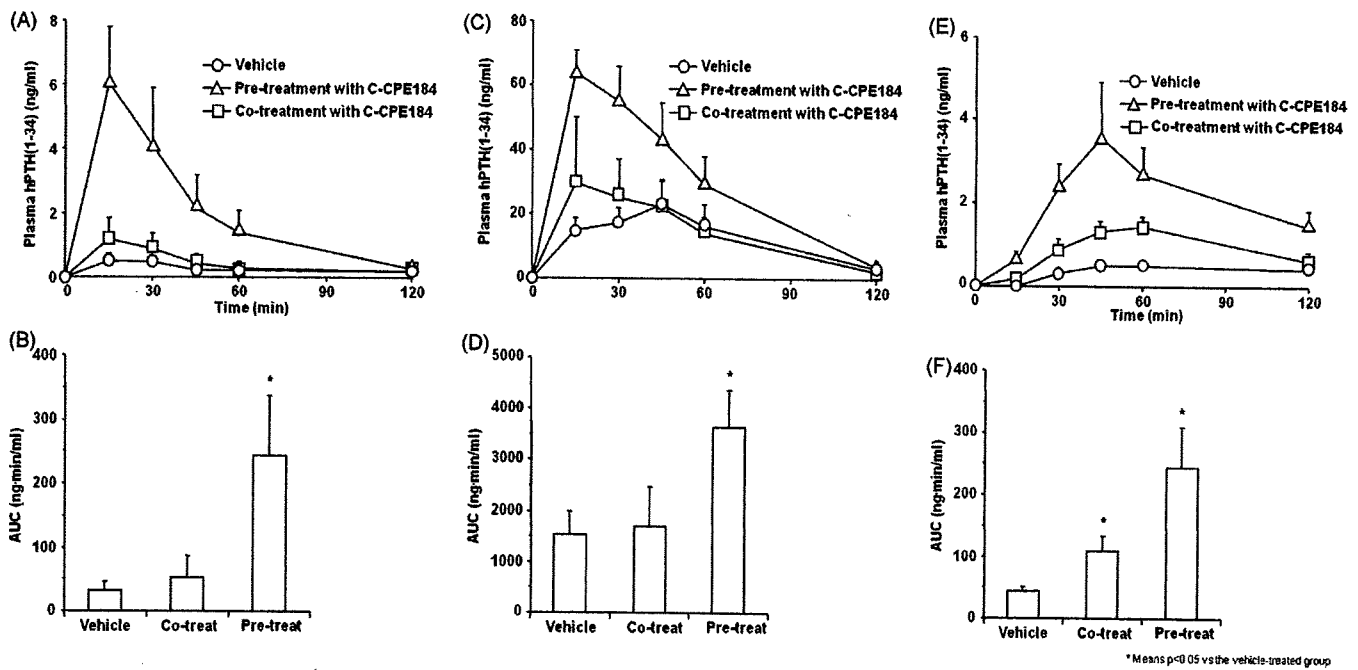
## 2.10. Statistical analysis

Data were analyzed by using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, and statistical significance was assigned at  $p < 0.05$ .

## 3. Results

### 3.1. Effects of C-CPE on jejunal, nasal and pulmonary absorption of a peptide drug

We previously found that a claudin-4 modulator, C-CPE184, is a novel type of absorption enhancer by using dextran as a model drug [21]. In the present study, we investigated whether the claudin-4 modulator enhances jejunal, nasal and pulmonary absorption of a peptide drug, hPTH(1-34). When hPTH(1-34) was administered with C-CPE184, C-CPE184 enhanced nasal absorption of hPTH(1-34) by 2.5-fold as compared to the vehicle-treated group. However, C-CPE184 did not enhance jejunal and pulmonary absorption of hPTH(1-34) (Fig. 1 and Table 1). Next, we examined whether pre-treatment of mucosa with C-CPE184 enhanced absorption of hPTH(1-34). When hPTH(1-34) was administered after 4 h of treatment with C-CPE184, the jejunal, nasal and pulmonary absorption of hPTH(1-34) was significantly



**Fig. 1.** Effect of C-CPE184 on mucosal absorption of hPTH(1-34) in rats. (A, B) Jejunal absorption of hPTH(1-34). Jejunum was co-treated with hPTH(1-34) (100  $\mu$ g) and C-CPE184 (20  $\mu$ g), or jejunum was treated with hPTH(1-34) 4 h after treatment with C-CPE184. (C, D) Pulmonary absorption of hPTH(1-34). hPTH(1-34) (150  $\mu$ g) was pulmonary administered with C-CPE184 (5  $\mu$ g) or 4 h after administration of C-CPE184. (E, F) Nasal absorption of hPTH(1-34). hPTH(1-34) (200  $\mu$ g) was nasally administered with C-CPE184 (2  $\mu$ g) or 4 h after administration of C-CPE184. Plasma hPTH(1-34) levels were measured at the indicated periods. Time-course changes in plasma hPTH(1-34) levels (A, C, E) and AUC from 0 to 120 min (B, D, F) were calculated. Data are mean  $\pm$  SE ( $n = 3-6$ ). Co-treat indicates co-treatment with both hPTH and C-CPE184, and Pre-treat indicates treatment with hPTH 4 h after C-CPE184-treatment. \*Significantly different from the vehicle-treated group ( $p < 0.05$ ).

**Table 1**

Parameters of mucosal absorption of hPTH(1-34) in C-CPE184-treated rats.

Treatments	Jejunum		Nasal		Pulmonary	
	Cmax (ng/ml)	BA (%) <sup>a</sup>	Cmax (ng/ml)	BA (%)	Cmax (ng/ml)	BA (%)
Vehicle	0.9 $\pm$ 0.3	0.4 $\pm$ 0.2	0.7 $\pm$ 0.1	0.3 $\pm$ 0.0	27.8 $\pm$ 6.5	14.6 $\pm$ 4.4
Co-treat	1.2 $\pm$ 0.6	0.6 $\pm$ 0.4	1.5 $\pm$ 0.2 <sup>*</sup>	0.8 $\pm$ 0.2 <sup>*</sup>	35.2 $\pm$ 18.9	14.9 $\pm$ 6.5
Pre-treat	6.0 $\pm$ 1.8 <sup>*</sup>	2.7 $\pm$ 0.9 <sup>*</sup>	3.6 $\pm$ 1.3	1.4 $\pm$ 0.4 <sup>*</sup>	67.6 $\pm$ 7.5 <sup>**</sup>	34.5 $\pm$ 6.6 <sup>*</sup>

<sup>a</sup> BA (%) = (AUC/Dose)/(AUC iv/Dose iv).

Data are means  $\pm$  SE.

<sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$ , as compared to vehicle-treated group.

increased 7.5-, 5.6- and 2.4-fold compared to the vehicle-treated group (Fig. 1 and Table 1).

### 3.2. Preparation of N-terminal-truncated C-CPE184-319 derivatives

The solubility of C-CPE184 is less than 0.3 mg/ml in PBS due to its hydrophobicity (Table 2). An increase in solubility without loss of claudin-4-modulating activity might improve the mucosal-absorption-enhancing activity of C-CPE184. Van Itallie et al. showed that the removal of the 10 N-terminal amino acids from C-CPE184 to yield C-CPE194 results in high solubility (10 mg/ml)

**Table 2**

Solubility of C-CPE184 and the N-terminal-truncated mutants.

Derivatives	Molecular size (kDa)	Solubility <sup>a</sup> (mg/ml)
C-CPE184	18.2	<0.3
C-CPE194	17.3	>10
C-CPE205	16.1	>4
C-CPE212	15.4	Insol. <sup>b</sup>
C-CPE219	14.7	Insol.
C-CPE224	14.2	Insol.

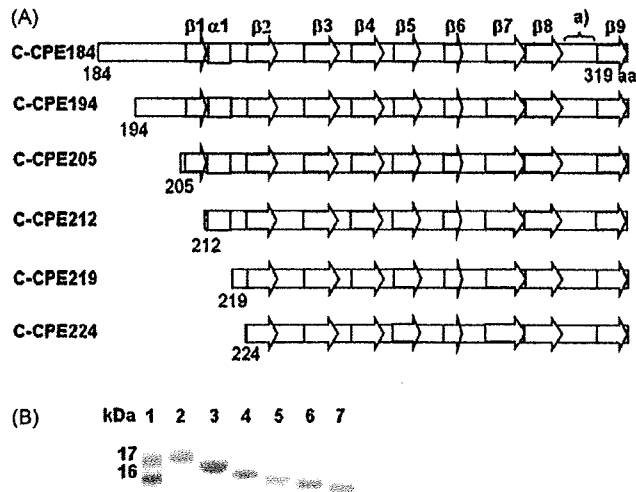
<sup>a</sup> Solvent is PBS.

<sup>b</sup> Insol., insoluble.

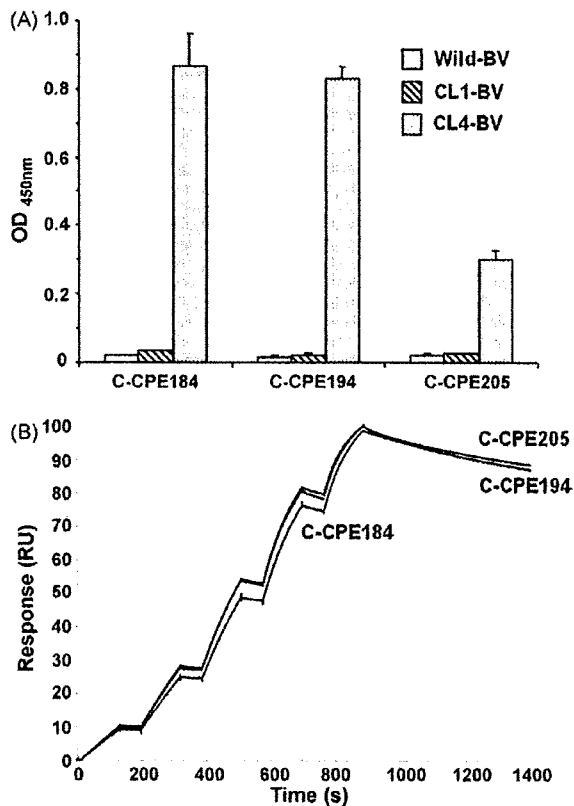
[26]. Although C-CPE194 is a claudin-4 binder, whether C-CPE194 modulates the TJ-barrier remains unclear. C-CPE194 contains nine  $\beta$ -sheets and one  $\alpha$ -helix, and its 16 C-terminal amino acids are believed to comprise the claudin-4-binding region (Fig. 2A) [26,29]. Based on this information, we prepared five different N-terminal-truncated C-CPE184 derivatives: C-CPE194, which lacks the 10 N-terminal amino acids; C-CPE205, which is truncated prior to the  $\beta$ 1-sheet; C-CPE212, which is truncated after the  $\beta$ 1-sheet; C-CPE219, which is truncated after the  $\alpha$ -helix; and C-CPE224, which is truncated before the  $\beta$ 2-sheet (Fig. 2A). The C-CPEs were expressed in *E. coli* (Fig. 2B). The solubility of C-CPE194 (>10 mg/ml) and C-CPE205 (>4 mg/ml) in PBS was greater than that of C-CPE184 (<0.3 mg/ml) (Table 2). However, C-CPE212, C-CPE219 and C-CPE224 formed solid inclusion bodies in *E. coli*, and these inclusion bodies could not be dissolved without 2 M urea. Therefore, further experiments were performed with C-CPE184, C-CPE194 and C-CPE205.

### 3.3. Characterization of C-CPE194 and C-CPE205

To study the interaction between the C-CPEs and claudin-4, we performed ELISA with claudin-4-displaying BV, as described previously [24]. When C-CPEs were added to claudin-4-displaying



**Fig. 2.** Preparation of C-CPEs. (A) Schematic structure of C-CPEs. Van Itallie et al. determined the 3-dimensional structure of C-CPE194 containing nine  $\beta$ -sheets and one  $\alpha$ -helix [26]. Based on the structural information, we designed five N-terminal truncated C-CPE184 derivatives. (B) CBB staining. C-CPEs were prepared and then purified by affinity chromatography. Lane 1, a maker for molecular weight; lane 2, C-CPE184; lane 3, C-CPE194; lane 4, C-CPE205; lane 5, C-CPE212; lane 6, C-CPE219; lane 7, C-CPE224.



**Fig. 3.** Interaction of C-CPEs with claudin. (A) ELISA. The immunoplate was coated with wild-type BV (Wild-BV), claudin-1-displaying BV (CL1-BV) or claudin-4-displaying BV (CL4-BV), and then C-CPEs were added to the well. C-CPEs bound to BVs were detected by the addition of anti-his tag antibody and a labeled secondary antibody. Data are means  $\pm$  SD ( $n = 3$ ). (B) SPR assay. Claudin-4 was immobilized on a CM5 sensor chip by the amine-coupling method. C-CPEs were injected sequentially at concentrations of 1.25, 2.5, 5, 10 and 20 nM. The association phase was monitored for 120 s at a flow rate of 10  $\mu$ l/min, and the dissociation phase was followed for 600 s at the same flow rate. The maximum values of response ( $R_{max}$ ) for all curves were compensated to 100 RU.

**Table 3**  
Binding kinetics of C-CPEs to claudin-4.

Derivatives	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_D$
C-CPE184	$5.96 \times 10^5$	$2.55 \times 10^{-4}$	429 pM
C-CPE194	$7.13 \times 10^5$	$3.24 \times 10^{-4}$	455 pM
C-CPE205	$7.67 \times 10^5$	$2.87 \times 10^{-4}$	374 pM

BV-adsorbed immunoplates, C-CPE194 and C-CPE205 bound to claudin-4-displaying BV but not mock BV or claudin-1-displaying BV (Fig. 3A). We performed SPR analysis to compare the affinities of C-CPEs to claudin-4. Claudin-4 proteins were fixed on the sensor chip, and C-CPEs were injected. Then, we measured the interaction between claudin-4 and C-CPEs. As shown in Fig. 3B and Table 3, C-CPE184, C-CPE194 and C-CPE205 had almost the same affinity to claudin-4 with  $K_D$  values of 429, 455 and 374 pM, respectively. The association and dissociation rates of C-CPE194 and C-CPE205 were also similar to those of C-CPE184. C-CPE184, C-CPE194 and C-CPE205 showed similar TJ-modulating activities in Caco-2 monolayer cells; their  $EC_{50}$  values were 0.49, 0.57 and 0.51  $\mu$ g/ml, respectively (Fig. 4A and Table 4). We performed in situ loop assays to examine the jejunal absorption of FD-4 by C-CPEs. C-CPE194 and C-CPE205 enhanced the jejunal absorption of FD-4 similar to C-CPE184 at 0.2 mg/ml (Fig. 4B–D). Treatment with C-CPE194 or C-CPE205 at 1.0 mg/ml yielded a greater and earlier absorption of FD-4 than treatment at 0.2 mg/ml (Fig. 4B, C). We could not test 1.0 mg/ml of C-CPE184 due to its low solubility.

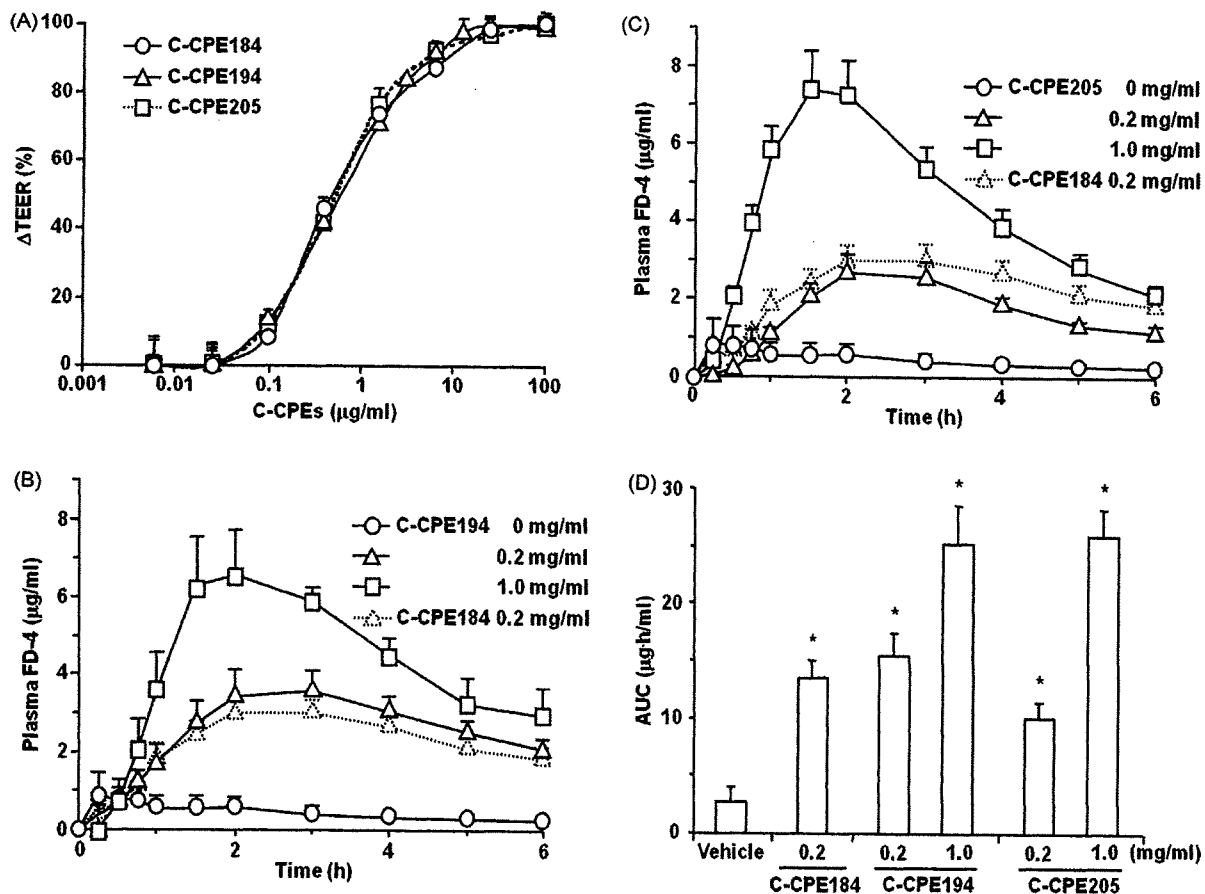
### 3.4. Jejunal and pulmonary absorption of hPTH(1-34) by co-treatment with C-CPE194

C-CPE194 enhanced the jejunal absorption of FD-4 to a similar extent as C-CPE184 and C-CPE205; C-CPE194 was also 30- and 3-fold more soluble than C-CPE184 and C-CPE205, respectively. C-CPE194 enhanced the jejunal absorption of hPTH(1-34) at 0.2 and 4.0 mg/ml (Fig. 5A). The AUC values were increased 11.0- and 18.4-fold as compared to the vehicle-treated group (Fig. 5B), and the  $C_{max}$  and BA of the jejunal absorption of hPTH(1-34) were also increased by C-CPE194 (Table 5). Additionally, the pulmonary absorption of hPTH(1-34) was enhanced by C-CPE194 ( $AUC = 3080.0 \pm 1994.3$  ng·min/ml in vehicle-treated group,  $AUC = 13,397.7 \pm 5830.1$  ng·min/ml in C-CPE194 (0.8 mg/ml)-treated group) (Fig. 5C, D). The  $C_{max}$  and BA of hPTH(1-34) were also increased by C-CPE194 (Table 5).

## 4. Discussion

Biologics are generally hydrophilic and poorly absorbed by the mucosa; therefore, many biologics are administered via injection. The development of a delivery system to allow biologics to pass across the epithelial barrier in mucosa is a pivotal issue for pharmaceutical therapy with biologics, since mucosal administration is needle-free, non-invasive, convenient and comfortable for patients [30,31]. We previously found that C-CPE184 enhanced jejunal absorption of dextran with a molecular mass of <10 kDa through its modulation of the claudin-4 barrier [21]. In the present study, we investigated the effect of a claudin-4 modulator on the mucosal absorption of a biologic, hPTH(1-34), and we found that a claudin-4 modulator is also a potent jejunal, nasal and pulmonary absorption enhancer of this biologic.

CPE is a 35-kDa polypeptide consisting of 319 amino acids [32]. The functional domain of CPE is divided into an N-terminal toxic domain and a C-terminal receptor-binding domain [33]. The receptor-binding fragments of CPE correspond to amino acids 169–319, 171–319, 184–319, 194–319 and 290–319 [20,26,33–35]. Among these fragments, only C-CPE184 and C-CPE194 have been



**Fig. 4.** Modulation of the TJ-barrier by C-CPEs. (A) Caco-2 cells were seeded on a BioCoat™. When TJ-barriers were developed and the cell sheets reached a plateau in their TEER value, C-CPEs were added to the wells from the basal side at the indicated concentration. After 18 h, the TEER was measured. The  $\Delta$ TEER was calculated as the ratio to reduced TEER values at 0 and 100  $\mu$ g/ml of C-CPE184 as 0% and 100%, respectively. Data are mean  $\pm$  SD ( $n = 3$ ). (B–D) Jejunal absorption of FD-4. Jejunum were treated with FD-4 and C-CPEs at the indicated concentration. Time-course changes in plasma FD-4 levels (B, C) and AUC from 0 to 6 h (D). Data are means  $\pm$  SE ( $n = 4$ –9). \*Significantly different from the vehicle-treated group ( $p < 0.05$ ).

proven to bind to claudin-4 [10,26], and a mucosal-absorption-enhancing effect was proven only for C-CPE184 [21]. The claudin-4 modulator C-CPE184 is a 400-fold more potent jejunal absorption enhancer of dextran as compared to a clinically used absorption enhancer, sodium caprate [21]. However, the low solubility of C-CPE184 (<0.3 mg/ml in PBS) has limited its applicability. This low solubility may result in the slow onset of TJ opening due to limiting the access of C-CPE184 to claudin. Last year, Van Itallie et al. made a breakthrough by truncating the N-terminal of C-CPE184 by 10 amino acids to yield C-CPE194 [26]. They found that C-CPE194 has affinity to claudin-4 and high solubility (>10 mg/ml); moreover, they determined the 3-dimensional structure of C-CPE194, which contains nine  $\beta$ -sheets and one  $\alpha$ -helix, and they suggested that the intervening surface loop spanning region 304–312 (located between the  $\beta$ 8 and  $\beta$ 9 sheets) may be a claudin-binding domain. Based on the structural data for C-CPE194, we prepared five N-terminal-truncated C-CPE184 derivatives: C-CPE194, C-CPE205, C-

CPE212 (without the  $\beta$ 1 sheet), C-CPE219 (without the  $\beta$ 1 sheet and  $\alpha$  helix), and C-CPE224 (without the  $\beta$ 1 sheet and  $\alpha$  helix). C-CPEs lacking the  $\beta$ 1 sheet are soluble in PBS containing 2 M Urea but insoluble in PBS. C-CPE184, C-CPE194 and C-CPE205 have almost the same kinetics parameters for binding to claudin-4 and the same TJ-barrier modulating activity (Table 3, Fig. 4A). Thus, the  $\beta$ 1 sheet appears to be critical for maintaining the structure of C-CPE, and the N-terminal region corresponding to amino acids 184–204 may not be involved in claudin-4 binding or TJ-barrier modulation.

Biologics must escape degradation by mucosal enzymes to be absorbed by the mucosa. C-CPE184 (0.2 mg/ml) did not enhance jejunal or pulmonary absorption of hPTH(1–34). However, when hPTH(1–34) was administered 4 h after treatment with C-CPE184, jejunal, pulmonary and nasal absorption was enhanced. Thus, hPTH(1–34) may be degraded in the jejunal and pulmonary mucosa before the enhancement of its absorption by co-administered C-CPE184. Indeed, another claudin-4 modulator, C-CPE194, which is 30-fold more soluble than C-CPE184, significantly enhanced the jejunal and pulmonary absorption of hPTH(1–34). These findings indicate that modulation of claudin-4 may be a potent strategy for mucosal-absorption enhancement of biologics.

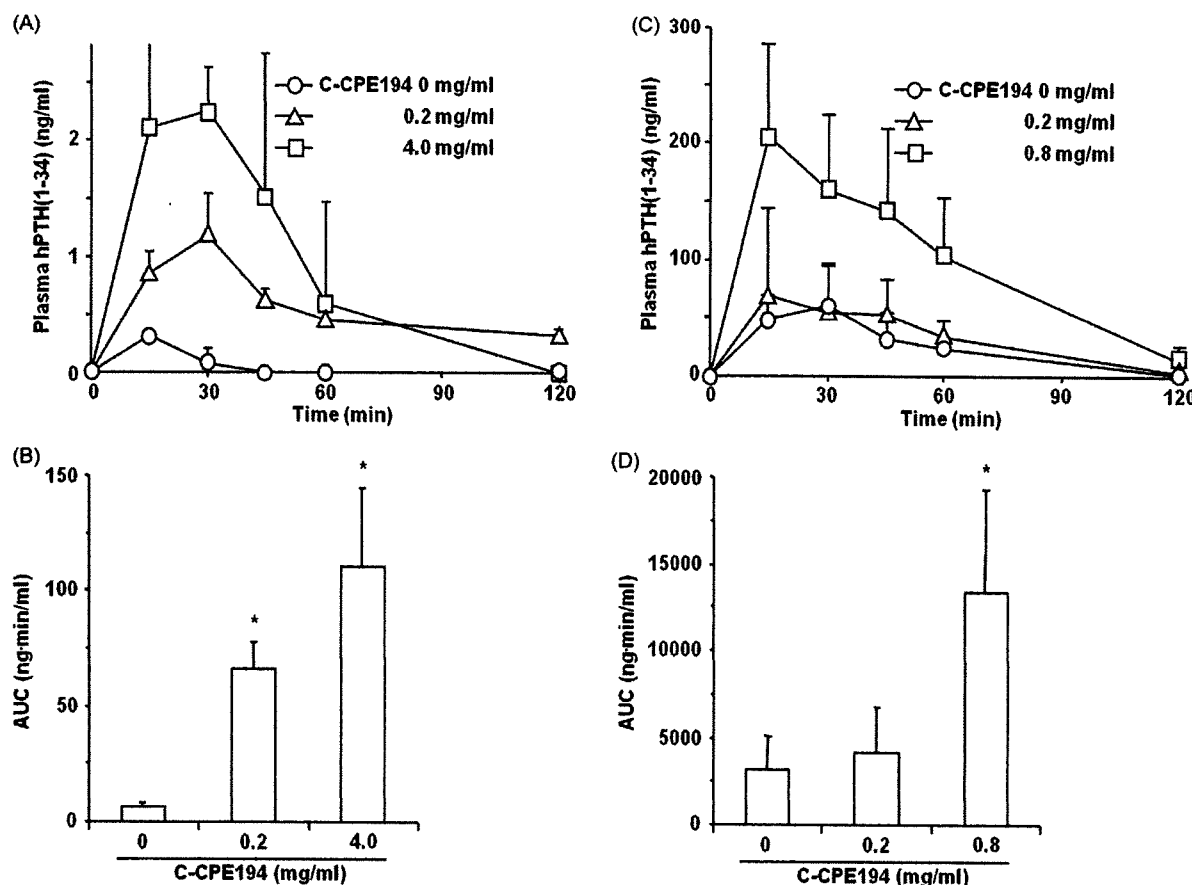
Meanwhile, a critical issue in the clinical application of the claudin-4 modulator as a mucosal-absorption enhancer is its safety. Problems with the safety of a claudin-4 modulator include the safety of a claudin-4 modulator in itself and the safety of the modulation of claudin-4, i.e., entry of unwanted substances by the

**Table 4**  
TJ-modulating activities of C-CPEs in Caco-2 cells.

Derivatives	EC50 values <sup>a</sup>
C-CPE184	0.49 $\mu$ g/ml
C-CPE194	0.57 $\mu$ g/ml
C-CPE205	0.51 $\mu$ g/ml

<sup>a</sup> The concentration of C-CPEs at which a 50% decrease in TEER value was observed in Fig. 4A.





**Fig. 5.** Mucosal absorption of hPTH(1-34) by a claudin-4 modulator. (A, B) Jejunal absorption of hPTH(1-34). Rat jejunum was treated with hPTH(1-34) (100  $\mu$ g) and C-CPE194 at the indicated doses. Time-course changes in plasma hPTH(1-34) (A) and AUC from 0 to 120 min (B) were analyzed. (C, D) Pulmonary absorption of hPTH(1-34). hPTH(1-34) (150  $\mu$ g) and C-CPE194 at the indicated doses were pulmonary administered, and time-course changes in plasma hPTH(1-34) concentration (C) and AUC from 0 to 120 min (D) were analyzed. Data are mean  $\pm$  SE ( $n = 3$ ). \*Significantly different from the vehicle-treated group ( $p < 0.05$ ).

opening of TJs. As mentioned above, C-CPEs are the only claudin-4 modulator. C-CPEs are polypeptide fragments of CPE consisting of more than 120 amino acids, and C-CPEs themselves may have antigenicity. Claudin-4 modulator significantly enhanced jejunal absorption of dextran with a molecular mass of less than 10 kDa [21], and C-CPEs (>14.2 kDa) may not be absorbed by the modulation of claudin-4. Moreover, *C. perfringens* are indigenous bacterium, and immunological tolerance may be induced. Thus, the antigenicity of C-CPE might be partly negligible. Because absorption enhancers would be used as an additive in drugs, they would be repeatedly administered. To avoid the risk of antigenicity, the development of a chemical compound-type or a 30–40 mer peptide-type of claudin modulator is needed. The determination of the 3-dimensional structure of claudin is also important for the theoretical development of promising claudin modulators. Ling et al. prepared a 12-mer peptide-type claudin-4 binder which did

not modulate TJs [36], and Van Itallie et al. determined the structure of C-CPE [26]. A peptide-type claudin modulator will be developed in the near future.

The other safety issue is the possible influx of unwanted substances that could be caused by the opening of TJs. C-CPE has demonstrated no damages to mucosal epithelial tissue in rat intestine [21]. Treatment of cells with C-CPE decreased the level of intracellular claudin-4 proteins paralleled by a disruption of the TJ-barrier [10]. Claudin contains the clathrin-sorting signal in its C-terminal intracellular domain, and claudin was often internalized [37,38]. Taken together, these results indicate that C-CPEs may disrupt the TJ-barrier, allowing the movement of solutes through the paracellular route. Do claudin modulators reversibly modulate the TJ-barrier and specifically regulate the movement of solutes? Disruption of the TJ-barrier by C-CPE is reversible, and the TJ-barrier gradually recovered after the removal of C-CPE [10]. The

**Table 5**  
Parameters of mucosal absorption of hPTH(1-34) in C-CPE 194-treated rats.

C-CPE194 (mg/ml)	Jejunum		C-CPE194 (mg/ml)	Pulmonary	
	Cmax (ng/ml)	BA (%) <sup>a</sup>		Cmax (ng/ml)	BA (%)
0	0.3 $\pm$ 0.0	0.1 $\pm$ 0.0	0	62.3 $\pm$ 32.8	26.5 $\pm$ 17.4
0.2	1.2 $\pm$ 0.4 <sup>*</sup>	0.8 $\pm$ 0.2 <sup>**</sup>	0.2	78.7 $\pm$ 66.1	34.1 $\pm$ 21.6
4.0	2.8 $\pm$ 0.2 <sup>*</sup>	1.3 $\pm$ 0.3 <sup>**</sup>	0.8	205.2 $\pm$ 79.4 <sup>*</sup>	100.6 $\pm$ 39.3 <sup>*</sup>

<sup>a</sup> BA (%) = (AUC/Dose)/(AUC iv/Dose iv).

Data are mean  $\pm$  SE.

<sup>\*</sup> $p < 0.05$ , <sup>\*\*</sup> $p < 0.01$  as compared to the vehicle-treated group.

quick recovery of TJ-barriers will need to be facilitated. One approach is the development of a quickly reversible claudin modulator. Another approach is the development of a claudin inducer for the combination of a claudin modulator and inducer. Another approach is the reduction of unwanted transport using the properties of claudins. Claudin comprises a multigene family consisting of 24 members. Claudin forms paired TJ strands by polymerization in a homomeric and heteromeric manner, and the claudin strands interact in a homotypic and heterotypic manner between adjacent cells [39,40]. TJ-barrier properties are believed to be determined by the combination and mixing ratios of claudin species [41]. Interestingly, the diversity of claudin may contribute to the regulation of specific solute movement through the paracellular route [17]. The expression profiles of claudin in mucosal epithelium exhibit heterogeneity [13–15,42]. The development of claudin modulators with solute and tissue specificity will reduce the non-specific influx of solutes caused by the modulation of TJs.

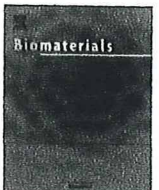
In summary, we found that claudin-4 modulator enhanced the jejunal, pulmonary and nasal absorption of a peptide drug. This report is the first to indicate that a claudin-4 modulator may be a mucosal-absorption enhancer of biologics.

### Acknowledgments

We thank M. Hoshino (Asubio Pharma), N. Kumagai (Asubio Pharma), K. Takashiba (Asubio Pharma), R. Okude (Osaka University) and Y. Nakano (GE Healthcare, Japan) for their excellent technical assistance. We also thank Drs Y. Horiguchi (Osaka University) and M. Furuse (Kobe University) for providing us C-CPE cDNA and claudin cDNA, respectively. This work was supported by a Grant-in-Aid for Scientific Research (21689006) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, by a Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan and by a grant from Kansai Biomedical Cluster project in Saito, which is promoted by the Knowledge Cluster Initiative of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

### References

- [1] Powell DW. Barrier function of epithelia. *Am J Physiol* 1981;241:G275–88.
- [2] Engel RH, Riggi SJ. Effect of sulfated and sulfonated surfactants on the intestinal absorption of heparin. *Proc Soc Exp Biol Med* 1969;130:879–84.
- [3] Tidball CS, Lipman RI. Enhancement of jejunal absorption of heparinoid by sodium ethylenediaminetetraacetate in the dog. *Proc Soc Exp Biol Med* 1962;111:713–5.
- [4] Aungst BJ. Intestinal permeation enhancers. *J Pharm Sci* 2000;89:429–42.
- [5] Deli MA. Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery. *Biochim Biophys Acta* 2009;1788:892–910.
- [6] Matsuhisa K, Kondoh M, Takahashi A, Yagi K. Tight junction modulator and drug delivery. *Expert Opin Drug Deliv* 2009;6:509–15.
- [7] Kondoh M, Yoshida T, Kakutani H, Yagi K. Targeting tight junction proteins-significance for drug development. *Drug Discov Today* 2008;13:180–6.
- [8] Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, et al. Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* 1993;123:1777–88.
- [9] Furuse M, Fujita K, Hiiiragi T, Fujimoto K, Tsukita S. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* 1998;141:1539–50.
- [10] Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, et al. *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. *J Cell Biol* 1999;147:195–204.
- [11] Furuse M, Hata M, Furuse K, Yoshida Y, Haratake A, Sugitani Y, et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol* 2002;156:1099–111.
- [12] Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* 2003;161:653–60.
- [13] Rahner C, Mitic LL, Anderson JM. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. *Gastroenterology* 2001;120:411–22.
- [14] Takano K, Kojima T, Go M, Murata M, Ichimiya S, Himi T, et al. HLA-DR- and CD11c-positive dendritic cells penetrate beyond well-developed epithelial tight junctions in human nasal mucosa of allergic rhinitis. *J Histochem Cytochem* 2005;53:611–9.
- [15] Wang F, Daugherty B, Keise LL, Wei Z, Foley JP, Savani RC, et al. Heterogeneity of claudin expression by alveolar epithelial cells. *Am J Respir Cell Mol Biol* 2003;29:62–70.
- [16] Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, Blasig IE. Structure and function of claudins. *Biochim Biophys Acta* 2008;1778:631–45.
- [17] Krause G, Winkler L, Piehl C, Blasig I, Piontek J, Muller SL. Structure and function of extracellular claudin domains. *Ann N Y Acad Sci* 2009;1165:34–43.
- [18] McClane BA, Hanna PC, Wnek AP. *Clostridium perfringens* enterotoxin. *Microb Pathog* 1988;4:317–23.
- [19] Fujita K, Katahira J, Horiguchi Y, Sonoda N, Furuse M, Tsukita S. *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. *FEBS Lett* 2000;476:258–61.
- [20] Katahira J, Inoue N, Horiguchi Y, Matsuda M, Sugimoto N. Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. *J Cell Biol* 1997;136:1239–47.
- [21] Kondoh M, Masuyama A, Takahashi A, Asano N, Mizuguchi H, Koizumi N, et al. A novel strategy for the enhancement of drug absorption using a claudin modulator. *Mol Pharmacol* 2005;67:749–56.
- [22] Wray C, Mao Y, Pan J, Chandrasena A, Piasta F, Frank JA. Claudin-4 augments alveolar epithelial barrier function and is induced in acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L219–27.
- [23] Suzuki Y, Yabuta M, Ohsuye K. High-level production of recombinant human parathyroid hormone 1–34. *Appl Environ Microbiol* 1998;64:526–9.
- [24] Saeki R, Kondoh M, Kakutani H, Tsunoda SI, Mochizuki Y, Hamakubo T, et al. A novel tumor-targeted therapy using a claudin-4-targeting molecule. *Mol Pharmacol* 2009;76:918–26.
- [25] Mitic LL, Unger VM, Anderson JM. Expression, solubilization, and biochemical characterization of the tight junction transmembrane protein claudin-4. *Protein Sci* 2003;12:218–27.
- [26] Van Itallie CM, Betts L, Smedley 3rd JG, McClane BA, Anderson JM. Structure of the claudin-binding domain of *Clostridium perfringens* enterotoxin. *J Biol Chem* 2008;283:268–74.
- [27] Karlsson R, Katsamba PS, Nordin H, Pol E, Myszkka DG. Analyzing a kinetic titration series using affinity biosensors. *Anal Biochem* 2006;349:136–47.
- [28] Myszkka DG. Improving biosensor analysis. *J Mol Recognit* 1999;12:279–84.
- [29] Takahashi A, Komiya E, Kakutani H, Yoshida T, Fujii M, Horiguchi Y, et al. Domain mapping of a claudin-4 modulator, the C-terminal region of C-terminal fragment of *Clostridium perfringens* enterotoxin, by site-directed mutagenesis. *Biochem Pharmacol* 2008;75:1639–48.
- [30] Sayani AP, Chien YW. Systemic delivery of peptides and proteins across absorptive mucosae. *Crit Rev Ther Drug Carrier Syst* 1996;13:85–184.
- [31] Song Y, Wang Y, Thakur R, Meidan VM, Michniak B. Mucosal drug delivery: membranes, methodologies, and applications. *Crit Rev Ther Drug Carrier Syst* 2004;21:195–256.
- [32] Czeizulin JR, Hanna PC, McClane BA. Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. *Infect Immun* 1993;61:3429–39.
- [33] Hanna PC, Wiecekowski EU, Mietzner TA, McClane BA. Mapping of functional regions of *Clostridium perfringens* type A enterotoxin. *Infect Immun* 1992;60:2110–4.
- [34] Hanna PC, Mietzner TA, Schoolnik GK, McClane BA. Localization of the receptor-binding region of *Clostridium perfringens* enterotoxin utilizing cloned toxin fragments and synthetic peptides. The 30 C-terminal amino acids define a functional binding region. *J Biol Chem* 1991;266:11037–43.
- [35] Kokai-Kun JF, McClane BA. Deletion analysis of the *Clostridium perfringens* enterotoxin. *Infect Immun* 1997;65:1014–22.
- [36] Ling J, Liao H, Clark R, Wong MS, Lo DD. Structural constraints for the binding of short peptides to claudin-4 revealed by surface plasmon resonance. *J Biol Chem* 2008;283:30585–9.
- [37] Ivanov AI, Nusrat A, Parkos CA. Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. *Mol Biol Cell* 2004;15:176–88.
- [38] Matsuda M, Kubo A, Furuse M, Tsukita S. A peculiar internalization of claudins, tight junction-specific adhesion molecules, during the intercellular movement of epithelial cells. *J Cell Sci* 2004;117:1247–57.
- [39] Furuse M, Furuse K, Sasaki H, Tsukita S. Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin-Darby canine kidney 1 cells. *J Cell Biol* 2001;153:263–72.
- [40] Furuse M, Sasaki H, Tsukita S. Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* 1999;147:891–903.
- [41] Furuse M, Tsukita S. Claudins in occluding junctions of humans and flies. *Trends Cell Biol* 2006;16:181–8.
- [42] Coyne CB, Gamblin TM, Boucher RC, Carson JL, Johnson LG. Role of claudin interactions in airway tight junctional permeability. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L1166–78.



## Mucosal vaccination using claudin-4-targeting

Hideki Kakutani<sup>a</sup>, Masuo Kondoh<sup>a,\*</sup>, Masahiro Fukasaka<sup>a</sup>, Hidehiko Suzuki<sup>a</sup>, Takao Hamakubo<sup>b</sup>, Kiyohito Yagi<sup>a,\*\*</sup>

<sup>a</sup> Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan

<sup>b</sup> Department of Molecular Biology and Medicine, Research Center for Advanced Science and Technology, The University of Tokyo, Meguro, Tokyo 153-8904, Japan

### ARTICLE INFO

#### Article history:

Received 12 January 2010

Accepted 19 March 2010

Available online xxx

#### Keywords:

Immunomodulation

Mucosa

Drug delivery

Epithelium

### ABSTRACT

Mucosa-associated lymphoid tissue (MALT) plays pivotal roles in mucosal immune responses. Efficient delivery of antigens to MALT is a critical issue for the development of mucosal vaccines. Although claudin-4 is preferentially expressed in MALT in the gut, a claudin-4-targeting approach for mucosal vaccination has never been developed. In the present study, we found that claudin-4 is expressed in nasal MALT, and we prepared a fusion protein of ovalbumin (OVA) as a model antigen with a claudin-4-binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) (OVA-C-CPE). Nasal immunization with OVA-C-CPE, but not a mixture of OVA and C-CPE, induced the production of OVA-specific serum IgG and nasal, vaginal and fecal IgA. Deletion of the claudin-4-binding region in OVA-C-CPE attenuated the induction of the immune responses. OVA-C-CPE immunization activated both Th1 and Th2 responses, and nasal immunization with OVA-C-CPE showed anti-tumor activity in mice inoculated with OVA-expressing thymoma cells. These results indicate that the claudin-4-targeting may be a potent strategy for nasal vaccination.

© 2010 Published by Elsevier Ltd.

### 1. Introduction

Each year, 17 million people die from infectious diseases worldwide, and 7 million people die from cancers worldwide ([http://www.globalhealth.org/infectious\\_diseases/](http://www.globalhealth.org/infectious_diseases/); <http://www.reuters.com/article/healthNews/idUSN1633064920071217>). Thus, the development of methods to prevent and treat infectious diseases and cancers is an important issue for healthcare worldwide. Vaccination against these diseases is a promising approach because of its low frequency of side effects and its great preventative and therapeutic effects. Vaccination strategies are classified as parenteral or mucosal.

**Abbreviations:** MALT, mucosa-associated lymphoid tissue; OVA, ovalbumin; C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin; OVA-C-CPE, fusion proteins of OVA and C-CPE; GALT, gut-associated lymphoid tissue; NALT, nasopharynx-associated lymphoid tissue; BALT, bronchus-associated lymphoid tissue; APC, antigen-presenting cell; FAE, follicle-associated epithelium; TJ, tight junction; CPE, *Clostridium perfringens* enterotoxin; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BV, budded baculovirus; FBS, fetal bovine serum; TBS, tris-buffered saline; IFN, interferon; IL, interleukin.

\* Corresponding author. Tel.: +81 6 6879 8196; fax: +81 6 6879 8199.

\*\* Corresponding author. Tel./fax: +81 6 6879 8195.

E-mail addresses: [masuo@phs.osaka-u.ac.jp](mailto:masuo@phs.osaka-u.ac.jp) (M. Kondoh), [yagi@phs.osaka-u.ac.jp](mailto:yagi@phs.osaka-u.ac.jp) (K. Yagi).

0142-9612/\$ – see front matter © 2010 Published by Elsevier Ltd.  
doi:10.1016/j.biomaterials.2010.03.047

Parenteral vaccination is effective for the elimination of infectious cells and cancer cells by the induction of systemic immune responses. Parenteral vaccines are administered by injections, which are invasive, painful, and have low levels of patient compliance; moreover, mucosal immunological defense is not induced. In contrast, mucosal vaccine elicits both mucosal and systemic immune responses, resulting in the prevention of infection on the mucosal surfaces and the elimination of pathological cells [1–3]. Mucosal administration is needle-free, less painful, and has improved patient compliance. Thus, mucosal vaccination appears to be an ideal vaccination strategy, although mucosally administered protein antigens are poorly immunogenic. Various approaches for the mucosal delivery of antigens have been investigated [4–6]. Mucosa-associated lymphoid tissues (MALTs) play pivotal roles in mucosal immunological responses [7,8]. MALTs comprise gut-associated lymphoid tissues (GALT), nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT). MALT contains lymphocytes, M cells, T cells, B cells and antigen-presenting cells (APCs), and the efficient delivery of antigens into MALT is essential for mucosal vaccinations [9]. Indeed, there have been several attempts to deliver antigens to MALT using microparticles, liposomes, saponins or chitosans [4–6].

Immunization at one mucosal surface can generate secretory IgA responses at other mucosal sites. Ideally, vaccination at a single site would provide both humoral and cell-mediated protection, not only



at the relevant mucosal surface, but also throughout the body [4]. In this regard, nasal vaccination has shown particular potential. Nasally administered vaccines induced mucosal IgA antibody responses in the salivary glands, respiratory tracts, genital tracts, and intestines [10–12]. The nasal route can also induce cytotoxic T lymphocytes in distant mucosal tissues including the female genital tract [13]. Additionally, nasal immunization produced greater systemic antibody responses than other mucosal immunization routes [12,14]. However, despite these encouraging characteristics, free antigens are usually unable to stimulate immune responses following intranasal administration due to their ineffective delivery to immune response-inducing sites [15]. Thus, the effective delivery of antigens to NALT is needed for the development of a potent nasal vaccine.

A single layer of epithelial cell sheet follicle-associated epithelium (FAE) covers NALT. FAE contains M cells, which are key antigen-sampling cells for the delivery of mucosally encountered antigens to the underlying APCs, and FAE plays a pivotal role in the mucosal immunological response [16–18]. Antigen delivery using a ligand for the FAE that covers NALT would be a potent strategy for the development of a mucosal vaccine. Epithelium has well-developed tight junctions (TJs) that seal the intercellular space on the epithelial cell sheets [19,20]. Occludin, claudin and junctional adhesion molecule are components of TJs [21]. Among these components, claudin-4 was preferentially expressed on the dome region of FAE in GALT [22]. We found that claudin-4 was also expressed in NALT (Fig. 1). These findings strongly indicate that claudin-4-targeting may be useful for mucosal vaccines; however, a mucosal vaccine that uses a claudin-4-binder has never been developed.

*Clostridium perfringens* enterotoxin (CPE) causes food poisoning in humans [23]. A receptor for CPE is claudin-4, and the C-terminal fragment of CPE (C-CPE) is a claudin-4-binder [24–26]. We previously prepared a claudin-4-targeting cytotoxic molecule by genetically fusing a cytotoxin with C-CPE [27,28]. In the present study, we investigated whether claudin-4-targeting is a potent strategy for mucosal vaccine using C-CPE-fused antigen protein.

## 2. Materials and methods

### 2.1. Animals

Female BALB/c mice and C57BL/6 mice (6–8 weeks old) were purchased from SLC, Inc. (Shizuoka, Japan). The mice were housed at  $23 \pm 1.5$  °C with a 12-h light/dark cycle and were allowed free access to standard rodent chow and water. After their arrival, the mice were allowed to adapt to their environment for at least 1 week before the experiments. The animal experiments were performed according to the guidelines of Osaka University.

### 2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total mRNA was extracted from NALT using Isogen (Nippongene, Toyama, Japan), and the mRNA was reverse-transcribed using an RNA PCR kit (AMV, Ver.3.0) according to the manufacturer's instructions (Takara, Kyoto, Japan). The polymerase chain reaction (PCR) amplification from the resultant cDNA was performed using primer pairs for claudin-4 (forward, 5'-tggatgaactgcgtggtg-3'; reverse, 5'-gggtgtagaagtcgcatgg-3') for 35 reaction cycles (94 °C, 45 s; 52 °C, 60 s; 72 °C, 30 s) or  $\beta$ -actin (forward, 5'-tagatgggcacagtgtggg-3'; reverse, 5'-ggcgtgatggggcatgg-3') for 30 reaction cycles (94 °C, 30 s; 58 °C, 60 s; 72 °C, 30 s). The amplified products were separated by electrophoresis on a 2% agarose gel and visualized with ethidium bromide.

### 2.3. Immunoblotting for claudin-4

NALT was lysed in a lysis buffer (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Triton X-100, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride). The lysates (10  $\mu$ g of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting with anti-claudin-4 (Zymed Laboratory, South San Francisco, CA) or anti- $\beta$ -actin antibodies (Sigma-Aldrich, St. Louis, MO). The immunoreactive bands were detected with a peroxidase-labeled secondary antibody followed by visualization with a chemiluminescence reagent (Amersham Bioscience, Piscataway, NJ).

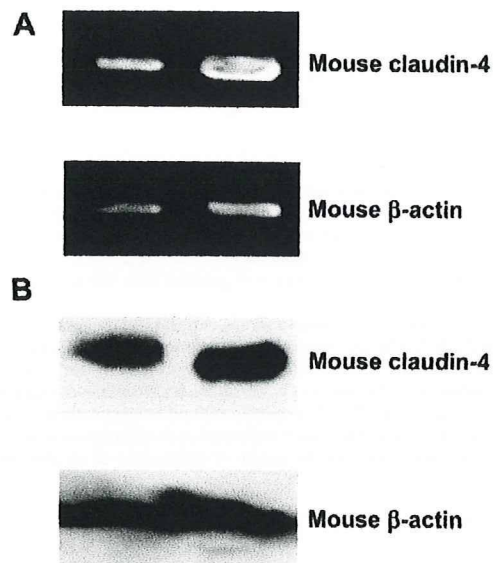


Fig. 1. Expression of claudin-4 in NALT. A) RT-PCR analysis. mRNA was isolated from NALT of mice, and expression of claudin-4 was assayed by RT-PCR. B) Immunoblot analysis. The lysate of NALT was subjected to SDS-PAGE, followed by western blotting with anti-claudin-4 Ab.  $\beta$ -actin was used as an internal control.

### 2.4. Preparation of OVA-C-CPE fusion proteins

We prepared expression plasmids encoding fusion proteins of OVA with C-CPE or C-CPE303, in which the claudin-4-binding C-terminal 16 amino acids of C-CPE were deleted [29]. Oligonucleotides containing a G4S linker and multiple cloning sites, including KpnI, SpeI, SmaI and PacI sites, were subcloned into NdeI-digested pET16b (Novagen, Darmstadt, Germany), pET-C-CPE and pET-C-CPE303 [30], resulting in pET-MCS and pET-MCS-C-CPEs. OVA cDNA was PCR amplified using pCMV Script/OVA (Kindly provided from Dr. S. Nakagawa, Osaka University, Japan) as a template, a forward primer (5'-gcggtaccatgggctccatggcgagc-3', KpnI site is underlined), and a reverse primer (5'-cccttaalftaaaggggaacacatctgcca-3', PacI site is underlined). The resulting OVA fragment was inserted into pET-MCS and pET-MCS-C-CPEs at the KpnI/PacI site, resulting in pET-OVA, pET-OVA-C-CPE and pET-OVA-C-CPE303. The OVA-fusion protein plasmids were transduced into *Escherichia coli* strain BL21 (DE3), and the production of OVA and OVA-C-CPEs was induced by the addition of isopropyl- $\beta$ -thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1 mM PMSF, 1 mM 2-mercaptoethanol, and 10% glycerol) supplemented with 8 M urea when necessary. The lysates were applied to HiTrap™ HP (GE Healthcare, Buckinghamshire, UK), and the fusion proteins were eluted with buffer A containing 100–500 mM imidazole. The solvent was exchanged with phosphate-buffered saline (PBS) using a PD-10 column (GE Healthcare), and the purified protein was stored at –80 °C until use. Purification of the fusion proteins was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-his-tag antibody. Protein assays were performed using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

### 2.5. Enzyme-linked immunosorbent assay (ELISA)

Budded baculovirus (BV) displaying mouse claudin-1 or -4 was prepared as described previously [28]. Briefly, the DNA fragments of claudin-1 or -4 were subcloned into the baculoviral transfer vector pFastBac1 (Invitrogen, Gaithersburg, MD). Recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen). Sf9 cells maintained in Grace's Insect medium containing 10% fetal bovine serum (FBS) at 27 °C were infected with the recombinant baculoviruses. After 70 h, the conditioned medium was recovered and centrifuged. The resultant pellets of the BV fraction were suspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail and then stored at 4 °C until use.

The BV displaying claudins was diluted with TBS and adsorbed to the wells of 96-well ELISA plates (Greiner Bio-One, Tokyo, Japan) overnight at 4 °C. The wells were blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 2 h at room temperature and the C-CPE, OVA-C-CPE or OVA-C-CPE303 was added. After 2-h incubation, the wells were washed and incubated with anti-his-tag antibody followed by a horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were detected using TMB peroxidase substrate at an absorbance of 450 nm.



## 2.6. Nasal immunization

Mice were nasally immunized with 10- $\mu$ l aliquots of OVA, a mixture of OVA and C-CPE, OVA-C-CPE or OVA-C-CPE303 at the indicated schedules. The doses of the proteins were equal to 5  $\mu$ g of OVA and 1.89  $\mu$ g of C-CPE.

## 2.7. OVA-specific antibody production

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 at 3000 $\times$ g for 10 min, and the resultant supernatants were used as fecal extracts. Vaginal and nasal mucosa were washed with 100 or 200  $\mu$ l of PBS, respectively.

The titers of OVA-specific antibody in serum, extracts and mucosal washes were determined by ELISA. Briefly, an immunoplate was coated with OVA (100  $\mu$ g/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 anti-mouse IgG, IgG1, IgG2a or IgA. The OVA-specific antibodies were detected using TMB peroxide substrate. End-point titers were expressed as the dilution ratio, which gave 0.1 above control values obtained for serum of naïve mice at an absorbance of 450 nm.

## 2.8. Cytokine ELISA

Serum interferon factor- $\gamma$  (IFN- $\gamma$ ) and Interleukin-13 (IL-13) were measured with an ELISA kit according to the manufacturer's protocol (R&D Systems, Inc., MN).

## 2.9. Cell cultures

A murine thymoma cell line EL4 (H-2<sup>b</sup>) was cultured in RPMI 1640 supplemented with 10% FBS. EG7-OVA cells (OVA-transfected EL4 cells) were maintained in RPMI 1640 containing 10% FBS in the presence of 400  $\mu$ g/ml of G418.

## 2.10. Anti-tumor activity

In an anti-tumor assay, female C57BL/6 mice (6–8 weeks) were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA-C-CPE or OVA-C-CPE303 once a week for 3 weeks. All non-vehicle immunizations contained equivalent amounts of OVA (5  $\mu$ g). Seven days after the last immunization, the mice were subcutaneously inoculated with  $1 \times 10^6$  EG7-OVA cells. Tumor growth was monitored by measuring two diameters, and the tumor volume was calculated as  $a \times b \times b/2$ , where  $a$  is the maximum diameter of the tumor and  $b$  is the minimum diameter of the tumor.

## 2.11. Statistical analysis

Results were analyzed by an analysis of variance (ANOVA) followed by the Dunnett multiple comparison test, and statistical significance was assigned at  $p < 0.05$ .

## 3. Results

### 3.1. Expression of claudin-4 in NALT

Nasal vaccine is a potent therapy for infectious diseases and cancers since nasal vaccination potentiates humoral and cellular immune responses throughout the body. NALT is the nasal lymphoid tissue, and effective delivery of antigens to NALT is critical for the development of mucosal vaccinations. A previous report showed that claudin-4 is expressed in GALT [22], whereas it is unclear whether claudin-4 is expressed in NALT. To investigate the expression of claudin-4 in NALT, NALT was isolated from mice, and the NALT lysate was subjected to RT-PCR and immunoblotting analyses. As shown in Fig. 1A and B, claudin-4 mRNA and protein were detected in NALT. These data indicate that claudin-4 binder may be a targeting molecule for NALT.

### 3.2. Preparation of claudin-4-targeting OVA

Claudin has low antigenicity, and there has been little success in the preparation of antibodies against the extracellular region of claudin. C-CPE corresponding to aa 184–319 at the C-terminal of CPE is a claudin-4-binder [24,25]. We previously prepared

a claudin-4-targeting cytotoxic molecule genetically fused with C-CPE [27]. To evaluate whether a claudin-4-targeting strategy is an effective method for mucosal vaccination, we genetically fused C-CPE with OVA, a popular model antigen for vaccination, to yield OVA-C-CPE (Fig. 2A). OVA-C-CPE was produced by *E. coli* and purified by affinity chromatography. Purification of the protein was confirmed by SDS-PAGE and immunoblotting (Fig. 2B). The molecular size was identical to the predicted size of 62 kDa for OVA-C-CPE. To evaluate the binding of OVA-C-CPE to claudin-4, we performed ELISA with a claudin-displaying BV-coated immunoplate. OVA-C-CPE or C-CPE was added to wells coated with wild-type BV, claudin-1-BV or claudin-4-BV. The bound proteins were detected using anti-his-tag antibody. Like C-CPE, OVA-C-CPE bound to claudin-4-BV but not wild-type BV or claudin-1-BV (Fig. 2C).

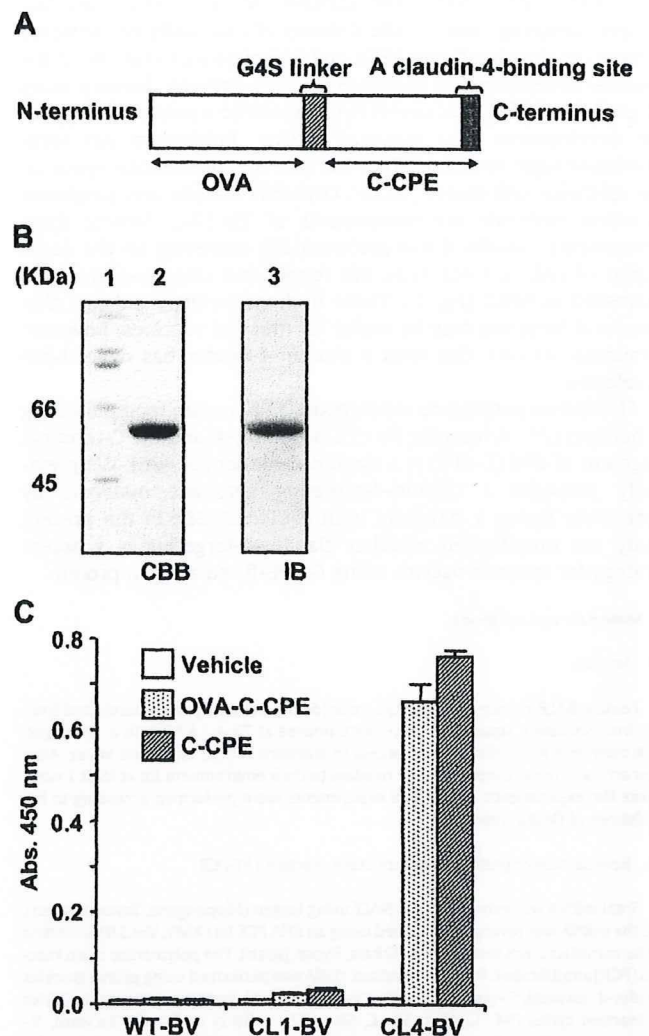


Fig. 2. Preparation of OVA-C-CPE. A) Schematic illustration of OVA-C-CPE. The claudin-4-binding site of C-CPE is located in the C-terminal 16 amino acids [29]. OVA was fused with C-CPE at the N-terminal of C-CPE, resulting in OVA-C-CPE. B) Purification of OVA-C-CPE. OVA-C-CPE was expressed in *E. coli* as a his-tagged protein and isolated by Ni-affinity chromatography. The purification of OVA-C-CPE was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB, left panel) and by immunoblotting with an anti-his-tag antibody (IB, right panel). Lane 1: molecular weight marker; lane 2, 3: OVA-C-CPE. The putative molecular mass of OVA-C-CPE is 62 kDa. C) Binding of OVA-C-CPE to claudin-4. Wild-type BV (WT-BV), BV displaying claudin-1 (CL1-BV) or -4 (CL4-BV) was adsorbed onto a 96-well immunoplate, and then vehicle, OVA-C-CPE or C-CPE was added to the well. OVA-C-CPE or C-CPE bound to BV was detected by an anti-his-tag Ab followed by horseradish peroxidase-labeled secondary Ab. C-CPE was used as a positive control for a claudin-4-binding. Data are means  $\pm$  SD ( $n = 4$ ).



### 3.3. Induction of OVA-specific humoral responses

To clarify whether claudin-4-targeting activates an immune response, we investigated antigen-specific humoral responses at both systemic and mucosal sites in mice that received nasally administered OVA-C-CPE. Mice received an intranasal administration of OVA, a mixture of OVA and C-CPE, or OVA-C-CPE fusion protein once a week for 3 weeks. Seven days after the last administration, we measured the OVA-specific serum IgG, nasal IgA, vaginal IgA and fecal IgA levels. As shown in Fig. 3A, the OVA-specific serum IgG responses were increased in mice immunized with OVA-C-CPE as compared to the mice immunized with OVA or a mixture of OVA and C-CPE. The OVA-specific IgA responses in nasal washes were greater from mice immunized with OVA-C-CPE than from mice immunized with OVA or a mixture of OVA and C-CPE (Fig. 3B). It is a superior character of mucosal vaccination that antigen-specific IgA responses were induced not only at the immunized site but also at remote mucosal surfaces [4]. As shown in Fig. 3C and D, nasal immunization with OVA-C-CPE activated vaginal and fecal OVA-specific IgA responses. The OVA-specific IgA responses did not occur in mice immunized with a mixture of OVA and C-CPE. These data suggest that fusion of OVA with C-CPE is critical for successful nasal vaccination.

We previously found that the C-terminal 16 amino acids of C-CPE are essential for claudin-4-binding [29]. To investigate the

involvement of claudin-4 in OVA-specific humoral responses in mice nasally immunized with OVA-C-CPE, we prepared OVA-C-CPE303, in which the claudin-4-binding region was deleted (Fig. 4A). Deletion of the 16 amino acid region attenuated the claudin-4-binding of OVA-C-CPE (Fig. 4B). OVA-specific serum IgG and nasal, vaginal and fecal mucosal IgA responses were also attenuated in mice immunized with OVA-C-CPE303 (Fig. 4C and D, 4E and 4F, respectively). No histological mucosal injury was found after nasal immunization with OVA-C-CPE (data not shown). These findings indicate that claudin-4-targeting may be involved in nasal vaccination by OVA-C-CPE.

### 3.4. Induction of Th1 and Th2 responses by OVA-C-CPE

Nasal immunization of antigen induced antigen-specific immune responses including Th1- and Th2-type responses [31,32]. We next investigated whether nasal immunization with OVA-C-CPE evoked Th1- or Th2-type responses. The OVA-specific IgG1 (a Th2 response) and IgG2a (a Th1 response) responses in the serum of mice nasally immunized with OVA-C-CPE were significantly enhanced compared to those of mice immunized with OVA alone or a mixture of OVA and C-CPE (Fig. 5A). Measurement of Th1 (IFN- $\gamma$ ) and Th2 (IL-13)-specific cytokines in splenocytes isolated from mice nasally immunized with OVA, a mixture of OVA and C-CPE, or OVA-C-CPE showed that nasal immunization with OVA-C-CPE increased both Th1 and Th2 cytokine production (Fig. 5B). Th1 and

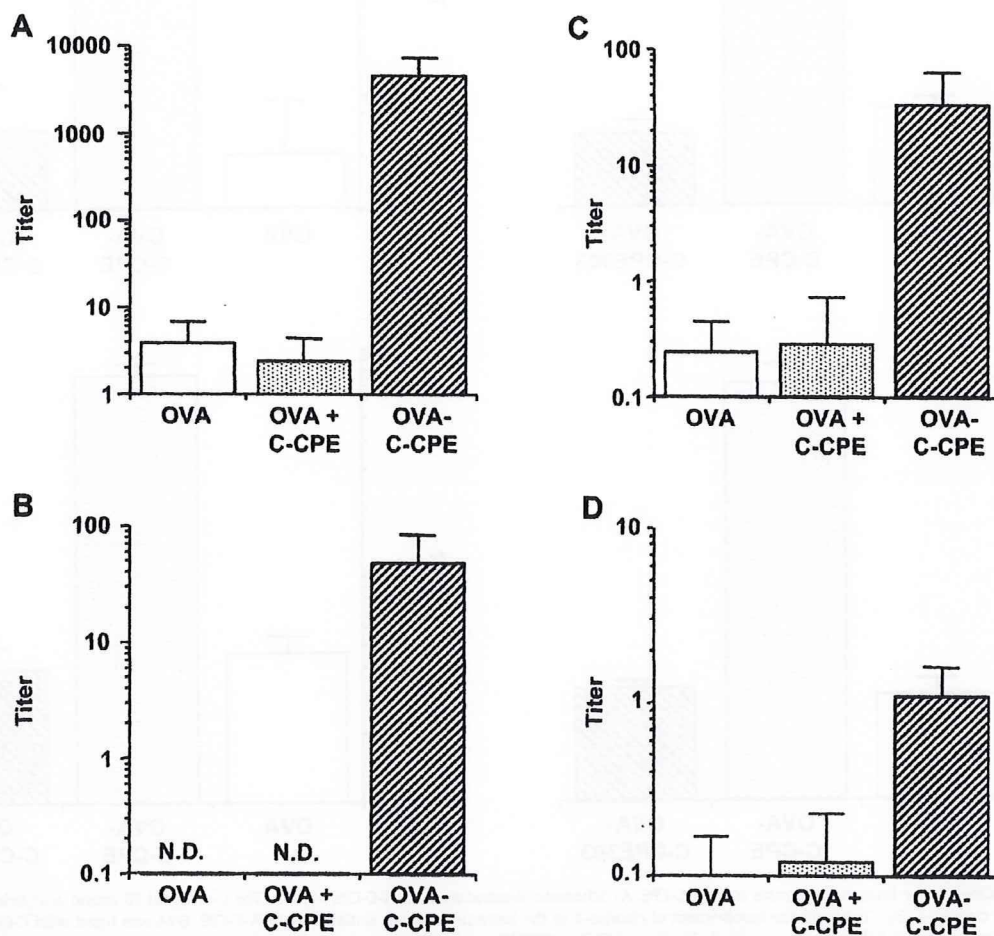
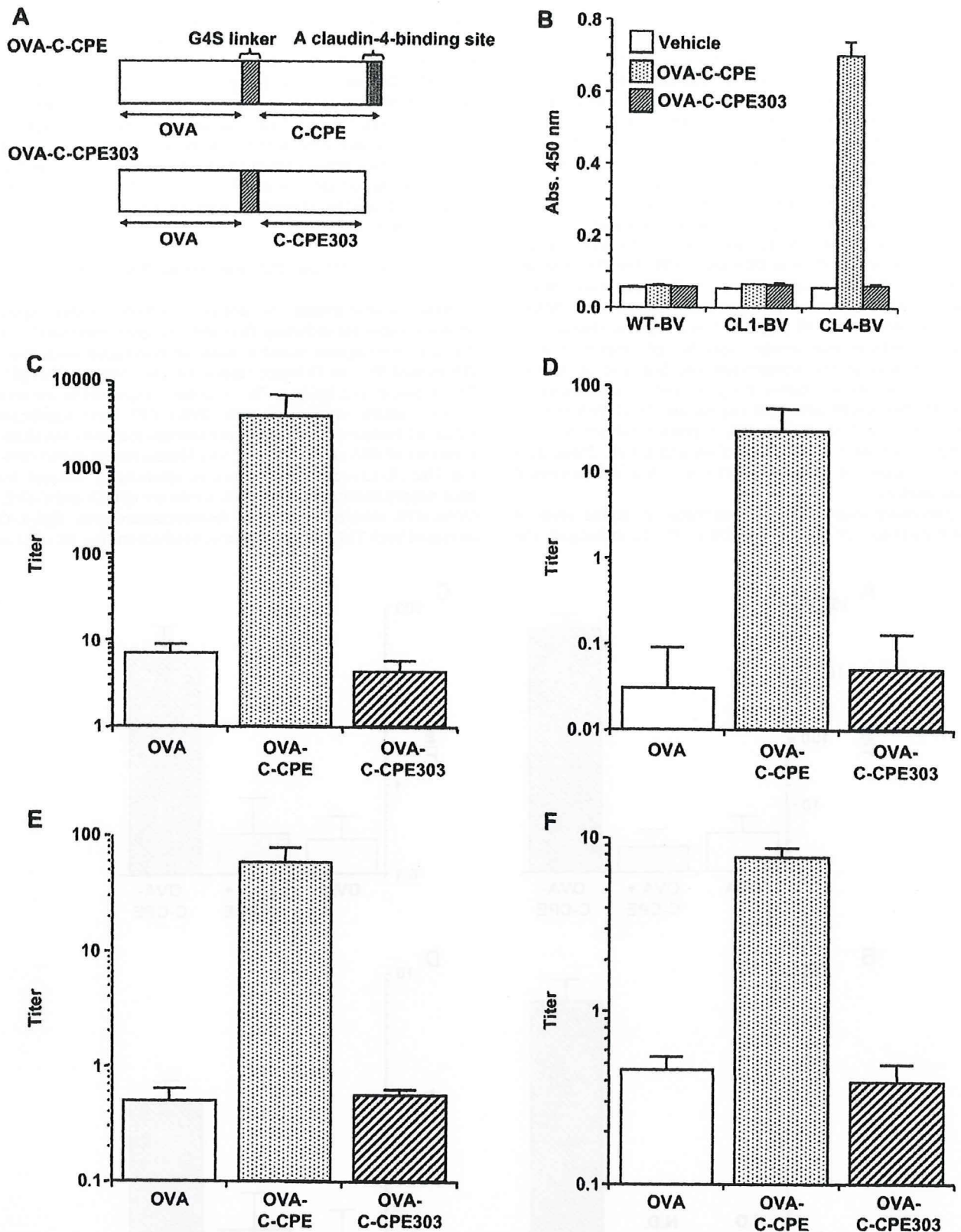
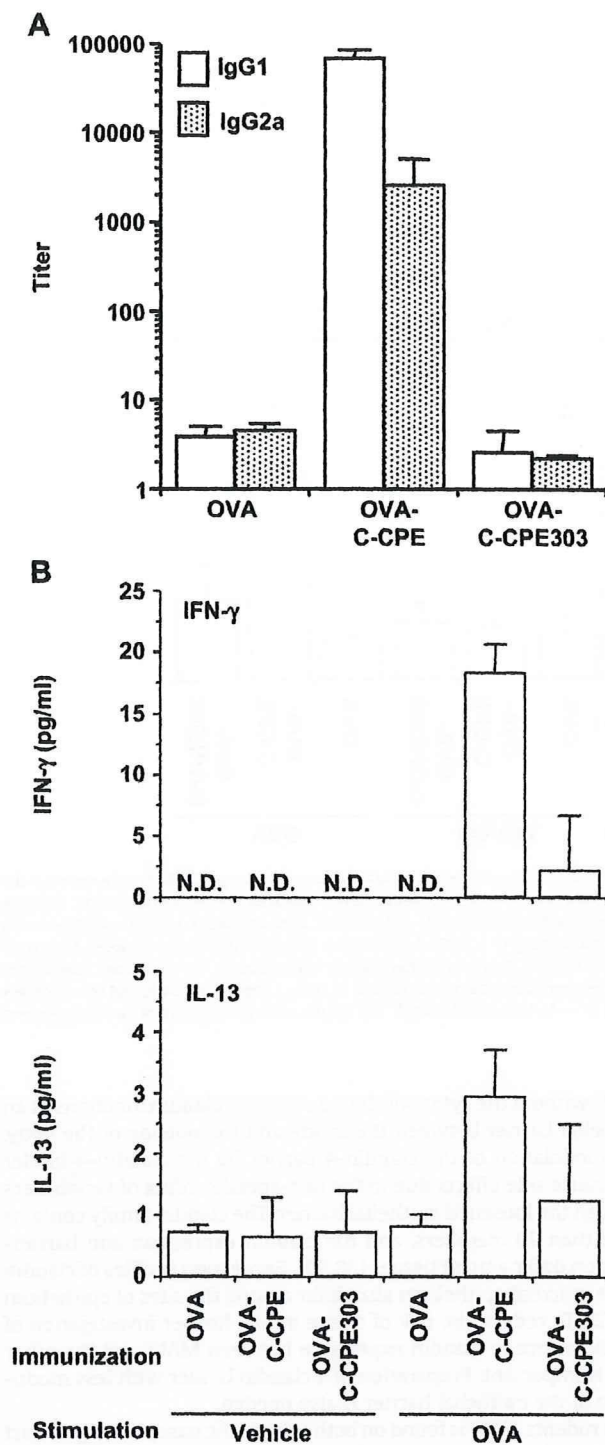


Fig. 3. Production of OVA-specific IgG and IgA by OVA-C-CPE. Mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, or OVA-C-CPE (5  $\mu$ g OVA) once a week for 3 weeks. Seven days after the last immunization, the levels of serum IgG (A), nasal IgA (B), vaginal IgA (C) and fecal IgA (D) were determined by ELISA. Data are means  $\pm$  SD ( $n = 4$ ). The results are representative of three independent experiments. N.D., not detected.



**Fig. 4.** Involvement of claudin-4 in the immune responses to OVA-C-CPE. **A**) Schematic illustration of OVA-C-CPE mutant. The C-terminal 16 amino acid-deleted C-CPE mutant (C-CPE303) did not bind to claudin-4 [29]. To clarify the involvement of claudin-4 in the immune response initiated by OVA-C-CPE, OVA was fused with C-CPE303, resulting in OVA-C-CPE303. **B**) Interaction of OVA-C-CPE303 with claudin-4. Binding of OVA-C-CPE303 to claudin-4 was investigated by ELISA with wild-type BV (WT-BV), claudin-1 or -4-displaying BV (CL1-BV, CL4-BV). **C**) Immune responses by OVA-C-CPE303. Mice were nasally immunized with OVA, OVA-C-CPE or OVA-C-CPE303 (5  $\mu$ g OVA) once a week for 3 weeks. Seven days after the last immunization, the levels of serum IgG (**C**), nasal IgA (**D**), vaginal IgA (**E**) and fecal IgA (**F**) were measured by ELISA. Data are means  $\pm$  SD ( $n = 4$ ). Data are representative of three independent experiments.





**Fig. 5.** Th1 and Th2 responses induced by OVA-C-CPE. Mice were nasally immunized with OVA, OVA-C-CPE or OVA-C-CPE303 (5  $\mu$ g of OVA) once a week for 3 weeks. Seven days after the last immunization, serum and splenocytes were collected. Serum IgG subclass (IgG1 and IgG2a) was determined by ELISA (A). The splenocytes isolated from the immunized mice were stimulated with vehicle or OVA (1 mg/ml) for 24 h, and the cytokines (IFN- $\gamma$  and IL-13) in the conditioned medium were measured by ELISA (B). Data are means  $\pm$  SD ( $n = 4$ ). N.D., not detected.

Th2 responses in IgG production and cytokines production were not observed in mice nasally immunized with OVA-C-CPE303 (Fig. 5A and B). These data indicate that claudin-4-targeting may be a potent method for mucosal vaccination.

### 3.5. Anti-tumor immune response induced by the claudin-4-targeting vaccine

To evaluate the immune responses induced by nasal vaccination with OVA-C-CPE, we performed an in vivo anti-tumor assay with EG7 thymoma cells, which are syngeneic tumor cells derived from OVA cDNA-transfected EL4 thymoma cells [33]. C57BL/6 mice were immunized with vehicle, OVA, a mixture of OVA and C-CPE, or OVA-C-CPE once a week for 3 weeks. Seven days after the last immunization, mice were challenged with EG7 thymoma cells. Tumor growth was monitored by calculating the average tumor volume. As shown in Fig. 6A, tumor growth was significantly suppressed in mice immunized with OVA-C-CPE, whereas the tumor growth was not suppressed in mice immunized with OVA or a mixture of OVA and C-CPE. Immunization with OVA-C-CPE303, in which the claudin-4-binding region was deleted, did not induce a protective immune response against tumor challenge. Immunization with OVA or OVA-C-CPE303 did not stimulate Th1- and Th2-immune responses including IgG1, IgG2a, IFN- $\gamma$  and IL-13 production; whereas immunization with OVA-C-CPE stimulated these immune responses (Fig. 6B and C). These data indicate that nasal immunization with a claudin-4-targeting vaccine may be useful for cancer therapy.

## 4. Discussion

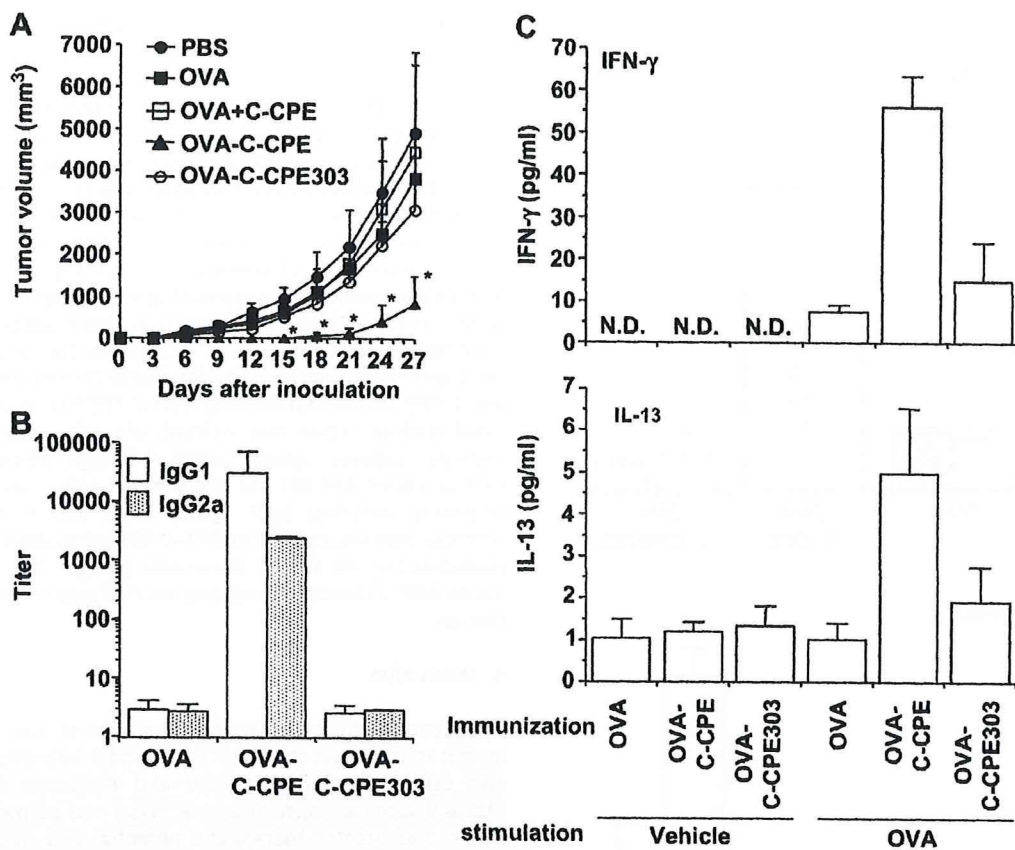
Recent progress in vaccine development has provided new insight into vaccine therapies for not only infectious diseases but also cancer, Alzheimer disease and Parkinson disease [3,34]. Mucosal vaccination, such as oral, nasal and pulmonary immunization, has greater therapeutic potential and increased patient comfort as compared to parenteral vaccination. The nasal cavity is the most promising site since it has low enzymatic activity and highly available immunoreactive sites; however, immunoresponses are not stimulated by intranasal administration of antigens [15,35–37]. Efficient delivery of antigens to NALT is critical for the development of nasal vaccines. In the present study, we found that intranasal immunization with antigen fused with a claudin-4-binder, C-CPE, stimulated humoral and mucosal immune responses and that these immune responses did not occur when the claudin-4-binding domain was deleted.

How does OVA-C-CPE activate immune responses? Claudin plays a pivotal role in the TJ-barrier in epithelium [38]. We previously found that C-CPE modulates the claudin-4 barrier and enhances mucosal absorption of dextran [30]. Activation of immune responses by OVA-C-CPE may be caused by modulation of the epithelial barrier in NALT, resulting in the uptake of OVA-C-CPE or its degradable product into NALT. OVA-C-CPE modulated the epithelial barrier in a human intestinal model of Caco-2 monolayer cells (data not shown). C-CPE enhanced jejunal absorption of dextran with a molecular mass of 4–20 kDa, and the integrity of the epithelial barrier in nasal mucosa was similar to that in jejunal mucosa [30,39]. OVA-C-CPE, which has a molecular mass of 62 kDa, may be poorly absorbed by nasal tissue. When OVA-C-CPE is degraded into fragments with a molecular mass of less than 20 kDa, the OVA fragment might be absorbed across nasal epithelium. A mixture of OVA and C-CPE did not induce an immune response, and deletion of the claudin-4-binding region in OVA-C-CPE attenuated the immune responses caused by nasal immunization with OVA-C-CPE. These findings indicate that targeting to claudin-4 rather than modulating the claudin-4 barrier by C-CPE is involved in the immune response to nasal vaccinations of OVA-C-CPE.

What cells are taken up OVA-C-CPE? NALT is covered by a unique epithelial layer known as FAE. Lymphocytes, T cells, B cells and APCs underlie the FAE. Antigen presentation to the

696  
697  
698  
699  
700  
701  
702  
703  
704  
705  
706  
707  
708  
709  
710  
711  
712  
713  
714  
715  
716  
717  
718  
719  
720  
721  
722  
723  
724  
725  
726  
727  
728  
729  
730  
731  
732  
733  
734  
735  
736  
737  
738  
739  
740  
741  
742  
743  
744  
745  
746  
747  
748  
749  
750  
751  
752  
753  
754  
755  
756  
757  
758  
759  
760





**Fig. 6.** Anti-tumor activity induced by immunization with OVA-C-CPE in an EG7 cancer model. **A)** Protective immune response against tumor challenge. C57BL/6 mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA-C-CPE, or OVA-C-CPE303 (5  $\mu$ g of OVA) once a week for 3 weeks. Seven days after the last immunization, the mice were injected s.c. on the right back with  $1 \times 10^6$  EG7 cells. The tumor volumes were calculated as described in the Materials and methods. Data are means  $\pm$  SD ( $n = 4$ ). The results are representative of two independent experiments. \*Significantly different from the vehicle-immunized group ( $P < 0.05$ ). **B, C)** Immune responses in the cancer model. Mice were nasally immunized with vehicle, OVA, OVA-C-CPE, or OVA-C-CPE303 (5  $\mu$ g of OVA) once a week for 3 weeks. Seven days after the last immunization, the serum and splenocytes were recovered. Serum IgG subclass (IgG1 and IgG2a) was determined by ELISA (**B**). The splenocytes were stimulated with vehicle or OVA (1 mg/ml) for 24 h, and the cytokines (IFN- $\gamma$  and IL-13) in the conditioned medium were measured by ELISA (**C**). Data are means  $\pm$  SD ( $n = 4$ ). N.D., not detected. The results are representative of two independent experiments.

immunocompetent cells by FAE is a trigger of mucosal immune responses [40,41]. Claudin-4 is expressed in the FAE of MALT [22]. Claudin-4 contains clathrin-sorting signal sequences in its C-terminal intracellular region [42,43]; thus, it may be taken up by clathrin-mediated endocytosis. Indeed, Matsuda et al. (2004) showed the endocytosis of claudins during the remodeling of TJs [44], and a C-CPE-fused molecule was intracellularly taken up [27]. OVA-C-CPE may be taken up into FAE followed by the presentation of antigens to the underlying immunocompetent cells. The FAE is enriched with specialized antigen-sampling epithelial cells known as M cells. M cells form an apparent pocket at the basal membrane site, and this pocket contains T cells, B cells, macrophages and dendritic cells. M cells deliver samples of foreign material by active transepithelial vesicular transport from the lumen directly to intraepithelial lymphoid cells and to subepithelial organized lymphoid tissue [6,16,40]. An antigen delivery system to M cells has been developed, and ligands for M cells, including a lectin, a peptide or a specific antibody, have been used for mucosal vaccination [45–48]. It has not been determined if claudin-4 is expressed in M cells and if OVA-C-CPE is taken up into M cells. Further investigation to clarify the mode of action of the claudin-4-targeting vaccine is needed.

Safety is essential for clinical application of the claudin-4-targeting vaccine. Histological injury was not detected after the administration of OVA-C-CPE. C-CPE is the receptor-binding domain

of CPE without the cytotoxic domain [24,49]. Claudin functions as an epithelial barrier between the inside and the outside of the body, and modulation of the claudin-4 barrier by the claudin-4-binder may cause side effects due to the non-specific influx of xenobiotics through the loosened epithelial barrier. The claudin family contains more than 20 members, and the claudin expression and barrier-function differ among tissues [38,50]. Expression profiles of claudin in the mucosal epithelium also differ among the sites of epithelium [51,52]. To reduce the risk of solute influx, further investigation of the difference in claudin expression between MALT and the other sites is important. Preparation of a claudin binder with less modulation of the epithelial barrier is also needed.

In rodents, NALT is found on both sides of the nasopharyngeal duct dorsal to the cartilaginous soft palate. Humans do not have NALT, except at an early age [53]; but, they possess oropharyngeal lymphoid tissues, including unpaired nasopharyngeal tonsils (adenoids) and bilateral tubular palatine, and lingual tonsils (Waldeyer's ring), which seem to correspond functionally to NALT [7,54]. The expression of claudin-4 in the human MALT, such as the tonsils and adenoids, should be investigated for the development of oral mucosal vaccine.

## 5. Conclusions

In the present study, we prepared C-CPE-fused OVA, and we found that the intranasal administration of the fusion protein increased not



only nasal IgA levels but also OVA-specific serum IgG, vaginal IgA and fecal IgA levels. Moreover, deletion of the claudin-4-binding region in the fusion protein caused the loss of immunomodulating activities. The claudin-4-targeting antigen immunization activated both Th1 and Th2 responses and showed anti-tumor activity in mice inoculated with OVA-expressing thymoma cells. This is the first report to indicate that claudin-4-targeting may be a promising strategy for the development of mucosal vaccines.

## Acknowledgements

We thank Drs Y. Horiguchi, S. Nakagawa (Osaka University), M. Furuse (Kobe University) and J. Kunisawa (The University of Tokyo) for providing C-CPE cDNA, OVA cDNA, claudin cDNA and useful comments, respectively. We also thank the all members of our laboratory for their useful comments and discussion. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (21689006), by a Health and Labor Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, by Takeda Science Foundation, by a Suzuken Memorial Foundation, by a grant from Kansai Biomedical Cluster project in Saito, which is promoted by the Knowledge Cluster Initiative of the Ministry of Education, Culture, Sports, Science and Technology, Japan, by a Research Grant for Promoting Technological Seeds from Japan Science and Technology Agency and the Japan Health Sciences Foundation.

## References

- Boyaka PN, Marinero M, Vancott JL, Takahashi I, Fujihashi K, Yamamoto M, et al. Strategies for mucosal vaccine development. *Am J Trop Med Hyg* 1999;60:35–45.
- Cardenas-Freytag L, Cheng E, Mirza A. New approaches to mucosal immunization. *Adv Exp Med Biol* 1999;473:319–37.
- Michels KB, zur Hausen H. HPV vaccine for all. *Lancet* 2009;374:268–70.
- Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol* 2006;6:148–58.
- Ryan EJ, Daly LM, Mills KH. Immunomodulators and delivery systems for vaccination by mucosal routes. *Trends Biotechnol* 2001;19:293–304.
- Yuki Y, Kiyono H. New generation of mucosal adjuvants for the induction of protective immunity. *Rev Med Virol* 2003;13:293–310.
- Kiyono H, Fukuyama S. NALT-versus Peyer's-patch-mediated mucosal immunity. *Nat Rev Immunol* 2004;4:699–710.
- Kunisawa J, Fukuyama S, Kiyono H. Mucosa-associated lymphoid tissues in the aerodigestive tract: their shared and divergent traits and their importance to the orchestration of the mucosal immune system. *Curr Mol Med* 2005;5:557–72.
- Kunisawa J, Nochi T, Kiyono H. Immunological commonalities and distinctions between airway and digestive immunity. *Trends Immunol* 2008;29:505–13.
- Imaoka K, Miller CJ, Kubota M, McChesney MB, Lohman B, Yamamoto M, et al. Nasal immunization of nonhuman primates with simian immunodeficiency virus p55gag and cholera toxin adjuvant induces Th1/Th2 help for virus-specific immune responses in reproductive tissues. *J Immunol* 1998;161:5952–8.
- Rudin A, Riise GC, Holmgren J. Antibody responses in the lower respiratory tract and male urogenital tract in humans after nasal and oral vaccination with cholera toxin B subunit. *Infect Immun* 1999;67:2884–90.
- Staats HF, Montgomery SP, Palker TJ. Intranasal immunization is superior to vaginal, gastric, or rectal immunization for the induction of systemic and mucosal anti-HIV antibody responses. *AIDS Res Hum Retroviruses* 1997;13:945–52.
- Gallichan WS, Rosenthal KL. Long-term immunity and protection against herpes simplex virus type 2 in the murine female genital tract after mucosal but not systemic immunization. *J Infect Dis* 1998;177:1155–61.
- Kozlowski PA, Williams SB, Lynch RM, Flanagan TP, Patterson RR, Cu-Uvin S, et al. Differential induction of mucosal and systemic antibody responses in women after nasal, rectal, or vaginal immunization: influence of the menstrual cycle. *J Immunol* 2002;169:566–74.
- Zinkernagel RM. Localization dose and time of antigens determine immune reactivity. *Semin Immunol* 2000;12:163–71.
- Neutra MR, Frey A, Kraehenbuhl JP. Epithelial M cells: gateways for mucosal infection and immunization. *Cell* 1996;86:345–8.
- Owen RL. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. *Gastroenterology* 1977;72:440–51.
- Owen RL, Piazza AJ, Ermak TH. Ultrastructural and cytoarchitectural features of lymphoreticular organs in the colon and rectum of adult BALB/c mice. *Am J Anat* 1991;190:10–8.
- Schneeberger EE, Lynch RD. Structure, function, and regulation of cellular tight junctions. *Am J Physiol* 1992;262:L647–61.
- Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* 2001;2:285–93.
- Mitic LL, Van Itallie CM, Anderson JM. Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. *Am J Physiol* 2000;279:G250–4.
- Tamagawa H, Takahashi I, Furuse M, Yoshitake-Kitano Y, Tsukita S, Ito T, et al. 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* 2003;83:1045–53.
- McClane BA, Chakrabarti G. New insights into the cytotoxic mechanisms of *Clostridium perfringens* enterotoxin. *Anaerobe* 2004;10:107–14.
- Katahira J, Inoue N, Horiguchi Y, Matsuda M, Sugimoto N. Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. *J Cell Biol* 1997;136:1239–47.
- Sonoda N, Furuse M, Sasaki H, Yonemura S, Katahira J, Horiguchi Y, et al. *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. *J Cell Biol* 1999;147:195–204.
- Van Itallie CM, Betts L, Smedley 3rd JG, McClane BA, Anderson JM. Structure of the claudin-binding domain of *Clostridium perfringens* enterotoxin. *J Biol Chem* 2008;283:268–74.
- Ebihara C, Kondoh M, Hasuiki N, Harada M, Mizuguchi H, Horiguchi Y, et al. Preparation of a claudin-targeting molecule using a C-terminal fragment of *Clostridium perfringens* enterotoxin. *J Pharmacol Exp Ther* 2006;316:255–60.
- Saeki R, Kondoh M, Kakutani H, Tsunoda S, Mochizuki Y, Hamakubo T, et al. A novel tumor-targeted therapy using a claudin-4-targeting molecule. *Mol Pharmacol* 2009;76:918–26.
- Takahashi A, Kondoh M, Masuyama A, Fujii M, Mizuguchi H, Horiguchi Y, et al. Role of C-terminal regions of the C-terminal fragment of *Clostridium perfringens* enterotoxin in its interaction with claudin-4. *J Control Release* 2005;108:56–62.
- Kondoh M, Masuyama A, Takahashi A, Asano N, Mizuguchi H, Koizumi N, et al. A novel strategy for the enhancement of drug absorption using a claudin modulator. *Mol Pharmacol* 2005;67:749–56.
- Yamamoto S, Kiyono H, Yamamoto M, Imaoka K, Fujihashi K, Van Ginkel FW, et al. A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. *Proc Natl Acad Sci USA* 1997;94:5267–72.
- Yanagita M, Hiroi T, Kitagaki N, Hamada S, Ito HO, Shimauchi H, et al. Nasopharyngeal-associated lymphoreticular tissue (NALT) immunity: fimbriae-specific Th1 and Th2 cell-regulated IgA responses for the inhibition of bacterial attachment to epithelial cells and subsequent inflammatory cytokine production. *J Immunol* 1999;162:3559–65.
- Moore MW, Carbone FR, Bevan MJ. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* 1988;54:777–85.
- Boche D, Nicol JA, Weller RO. Immunotherapy for Alzheimer's disease and other dementias. *Clin Neuropharmacol* 2006;29:22–7.
- Dahl R, Mygind N. Anatomy, physiology and function of the nasal cavities in health and disease. *Adv Drug Deliv Rev* 1998;29:3–12.
- Ugwoke MI, Agu RU, Verbeke N, Kinget R. Nasal mucoadhesive drug delivery: background, applications, trends and future perspectives. *Adv Drug Deliv Rev* 2005;57:1640–65.
- Ugwoke MI, Verbeke N, Kinget R. The biopharmaceutical aspects of nasal mucoadhesive drug delivery. *J Pharm Pharmacol* 2001;53:3–21.
- Furuse M, Tsukita S. Claudins in occluding junctions of humans and flies. *Trends Cell Biol* 2006;16:181–8.
- Rojanasakul Y, Wang LY, Bhat M, Glover DD, Malanga CJ, Ma JK. The transport barrier of epithelia: a comparative study on membrane permeability and charge selectivity in the rabbit. *Pharm Res* 1992;9:1029–34.
- Kraehenbuhl JP, Neutra MR. Molecular and cellular basis of immune protection of mucosal surfaces. *Physiol Rev* 1992;72:853–79.
- Neutra MR, Mantis NJ, Kraehenbuhl JP. Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat Immunol* 2001;2:1004–9.
- Bonifacino JS, Traub LM. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem* 2003;72:395–447.
- Ivanov AI, Nusrat A, Parkos CA. Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. *Mol Biol Cell* 2004;15:176–88.
- Matsuda M, Kubo A, Furuse M, Tsukita S. A peculiar internalization of claudins, tight junction-specific adhesion molecules, during the intercellular movement of epithelial cells. *J Cell Sci* 2004;117:1247–57.
- Higgins LM, Lambkin I, Donnelly G, Byrne D, Wilson C, Dee J, et al. In vivo phage display to identify M cell-targeting ligands. *Pharm Res* 2004;21:695–705.



- 1021 [46] Manocha M, Pal PC, Chitralakha KT, Thomas BE, Tripathi V, Gupta SD, et al.  
1022 Enhanced mucosal and systemic immune response with intranasal immunization  
1023 of mice with HIV peptides entrapped in PLG microparticles in combination with  
1024 Ulex Europaeus-I lectin as M cell target. *Vaccine* 2005;23:5599–617.
- 1025 [47] Nochi T, Yuki Y, Matsumura A, Mejima M, Terahara K, Kim DY, et al. A  
1026 novel M cell-specific carbohydrate-targeted mucosal vaccine effectively  
1027 induces antigen-specific immune responses. *J Exp Med* 2007;204:  
1028 2789–96.
- 1029 [48] Wang X, Kochetkova I, Haddad A, Hoyt T, Hone DM, Pascual DW. Transgene  
1030 vaccination using Ulex europaeus agglutinin I (UEA-1) for targeted mucosal  
1031 immunization against HIV-1 envelope. *Vaccine* 2005;23:3836–42.
- [49] Hanna PC, Wieckowski EU, Mietzner TA, McClane BA. Mapping of functional  
regions of *Clostridium perfringens* type A enterotoxin. *Infect Immun*  
1992;60:2110–4.
- [50] Morita K, Furuse M, Fujimoto K, Tsukita S. Claudin multigene family encoding  
four-transmembrane domain protein components of tight junction strands.  
*Proc Natl Acad Sci USA* 1999;96:511–6.
- [51] Chiba H, Osanai M, Murata M, Kojima T, Sawada N. Transmembrane proteins  
of tight junctions. *Biochim Biophys Acta* 2008;1778:588–600.
- [52] Rahner C, Mitic LL, Anderson JM. Heterogeneity in expression and subcellular  
localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut.  
*Gastroenterology* 2001;120:411–22.
- [53] Debertain AS, Tschernig T, Tonjes H, Kleemann WJ, Troger HD, Pabst R. Nasal-  
associated lymphoid tissue (NALT): frequency and localization in young  
children. *Clin Exp Immunol* 2003;134:503–7.
- [54] Koornstra PJ, de Jong FJ, Vlek LF, Marres EH, van Breda Vriesman PJ. The  
Waldeyer ring equivalent in the rat. A model for analysis of oronasophar-  
yngeal immune responses. *Acta Otolaryngol* 1991;111:591–9.

