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A Novel Screening System for Claudin Binder Using Baculoviral Display

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Abstract

Recent progress in cell biology has provided new insight into the claudin (CL) family of integral membrane proteins, which contains more than 20 members, as a target for pharmaceutical therapy. Few ligands for CL have been identified because it is difficult to prepare CL in an intact form. In the present study, we developed a method to screen for CL binders by using the budded baculovirus (BV) display system. CL4-displaying BV interacted with a CL4 binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), but it did not interact with C-CPE that was mutated in its CL4-binding region. C-CPE did not interact with BV and CL1-displaying BV. We used CL4-displaying BV to select CL4-binding phage in a mixture of a scFv-phage and C-CPE-phage. The percentage of C-CPE-phage in the phage mixture increased from 16.7% before selection to 92% after selection, indicating that CL-displaying BV may be useful for the selection of CL binders. We prepared a C-CPE phage library by mutating the functional amino acids. We screened the library for CL4 binders by affinity to CL4-displaying BV, and we found that the novel CL4 binders modulated the tight-junction barrier. These findings indicate that the CL-displaying BV system may be a promising method to produce a novel CL binder and modulator.

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Introduction

Tight junctions (TJ) are intercellular adhesion complexes in epithelial and endothelial cells; TJs are located in the most apical part of the complexes [1]. TJs have a barrier function and a fence function [2–4]. TJs contribute to epithelial and endothelial barrier functions by restricting the diffusion of solutes through the paracellular pathway. TJs maintain cellular polarity by preventing the free movement of membrane proteins between the apical and basal membranes [5]. Loss of cell-cell adhesion and cellular polarity commonly occurs in the early stages of cancer [6]. Modulation of the TJ barrier function can be a method to enhance drug absorption, and TJ components exposed on the surface of cancer cells can be a target for cancer therapy.

Biochemical analyses of TJs have identified TJ components, such as occludin, claudins (CLs) and junction adhesion molecule [7]. The CL family contains more than 20 integral tetra-transmembrane proteins that play pivotal roles in the TJ barrier and fence functions. CL1-deficient mice lack the epidermal barrier, while CL5-deficient mice lack the blood-brain barrier [8,9], indicating that the regulation of the TJ barrier by modulation of CLs may be a promising method for drug delivery. *Clostridium perfringens* enterotoxin (CPE) causes food poisoning in humans [10]. An interaction between the C-terminal domain of CPE (C-CPE) with CL4 deregulates the TJ barrier [11,12]. We previously found that C-CPE enhances jejunal absorption through its interaction with CL4, indicating that a CL binder is a potent drug-delivery system [13].

The majority of lethal cancers are derived from epithelial tissues [14]. Malignant tumor cells frequently exhibit abnormal TJ function, followed by the deregulation of cellular polarity and intercellular contact, which is commonly observed in both advanced tumors and the early stages of carcinogenesis [6]. Some CLs are overexpressed in various types of cancers. For example, CL3 and CL4 are overexpressed in breast, prostate, ovarian, pancreatic and gastric cancers. CL1, CL7, CL10 and CL16 are overexpressed in colon, gastric, thyroid and ovarian cancers, respectively [15,16]. These findings indicate that the CLs may be a target molecule for cancer therapy. A receptor for CPE is CL4 [11,12]. CPE has anti-tumor activity against human pancreatic and ovarian cancers without side effects [17,18]. The CLs binders will be useful for cancer-targeting therapy.

As above, recent investigations of CLs provide new insight into their use as pharmaceutical agents; for example, a CL binder may be used in drug delivery and anti-tumor therapy. Selection of a CL binder by using a recombinant CL protein is a putative method to

prepare a CL binder. However, CLs are four-transmembrane proteins with high hydrophobicity; there has been little success in the preparation of intact CL protein. Recently, a novel type of protein expression system that uses baculovirus has been developed. Membrane proteins are displayed on the budded baculovirus (BV) in their active form [19–21], indicating that the BV system may be useful for the preparation of a CL binder. In the present study, we investigated whether a CL binder was screened by using a CL-displaying BV.

Results

Preparation of CL4-displaying BV

C-CPE is the only known CL binder and modulator [12,13,22]. C-CPE has affinity to CL4 in a nanomolar range [23]. We chose C-CPE and CL4 as models of the CL binder and CL, respectively. Several reports indicate that membrane proteins expressed on the surface of BV are in an intact form [19–21]. To check the expression of CL4 on the BV, we performed immunoblot analysis of the lysate of CL4-BV against CL4. As shown in Fig. 1A, CL4 was detected in the virus lysates. To determine if the CL4 expressed on the virus has an intact form, we performed enzyme-linked immunosorbent assay (ELISA) with CL4-BV-coated immunoplates. C-CPE binds to the extracellular loop domain of CL4 [23]. After the addition of C-CPE to the CL4-BV-coated plate, the C-CPE bound to the CL4-BV-coated plate was detected by anti-his-tag antibody, followed by incubation with horseradish peroxidase-labeled antibody. C-CPE was dose-dependently bound to CL4-BV, whereas C-CPE did not interact with wild-BV (Fig. 1B). Deletion of the CL4-binding region (C-CPE303) attenuated the interaction of C-CPE with CL4-BV (Fig. 1C). Together, these results indicate that the CL4 displayed on BV may have an intact extracellular loop region.

Selection of C-CPE-phage by using CL4-BV

We next examined the interaction between C-CPE-phage and CL4-BV. As shown in Fig. 2A, C-CPE-phage bound to CL4-BV but not to wild-BV, and a scFv-phage did not bind to CL4-BV. To determine if CL-BV can be used to select CL binders, we prepared a mixture of C-CPE-phage and scFv-phage at a ratio of 2:10 and used CL4-BV to select CL4-binding phage in the mixtures. The amount of C-CPE-phage was increased to 11 of 12 clones in the mixture (Fig. 2B), indicating that CL-BV may be useful in the preparation of CL binders.

We previously found that each substitution of S304, S305, S307, N309, S313 and K318 with alanine increased the binding of C-CPE to CL4 [24]. Here, we prepared a phage library for C-CPE by randomly changing the functional 6 amino acids to any of the 20 amino acids. To confirm the diversity of the library, we checked the sequences of 17 randomly isolated clones. Each of the 17 clones had a different sequence, indicating that the library has a diverse population of C-CPE mutants (Table 1).

Then, we screened the CL4-binding phage by their affinity to CL4-BV. After addition of the C-CPE library to CL4-BV-adsorbed tubes, the CL4-BV-bound phages were recovered (1st screening). We repeated this screening process two more times (2nd screening and 3rd screening). If the number of CL4-bound phage is increased during the screening, the ratio of the incubated phage titers to the recovered phage titers will increase. As shown in Fig. 3A, the ratio was increased during screening from 4.5×10^{-7} to 5.5×10^{-5}, indicating that the screening system for CL4 binders may work. Indeed, the number of monoclonal phage clones with high affinity to CL4-BV was increased after the 3rd screening compared with that after the 2nd screening (Fig. 3B).

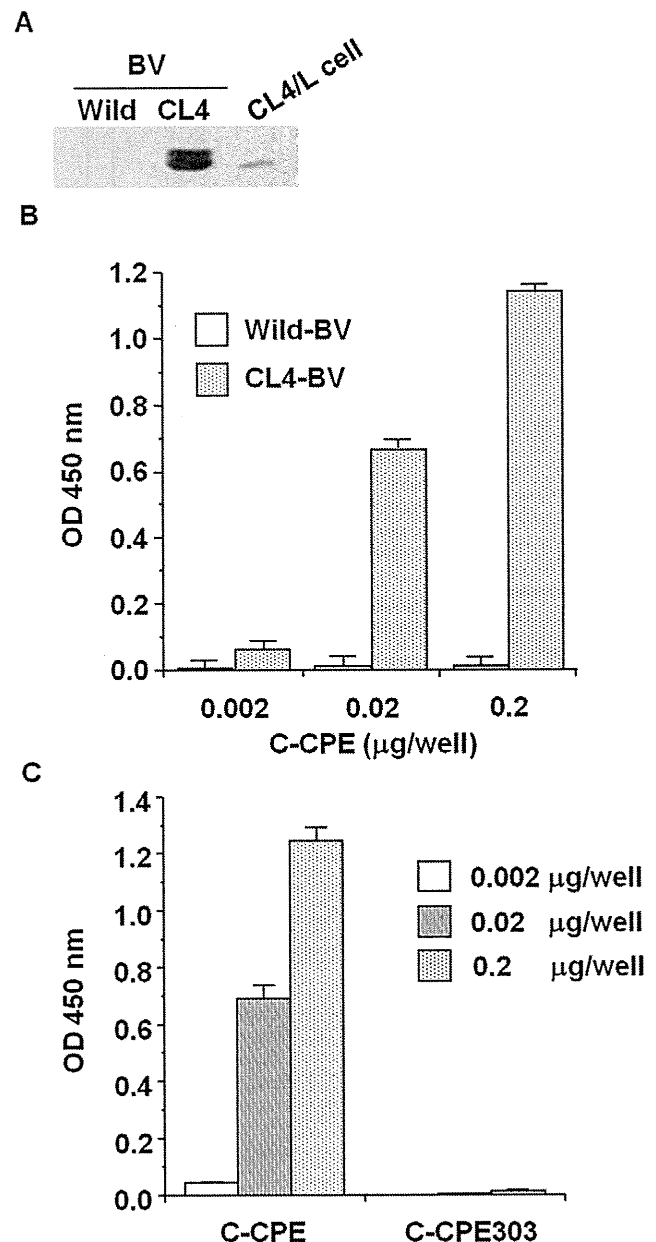

Figure 1. Preparation of CL4-displaying BV. A) Immunoblot analysis. Wild-BV and CL4-BV (0.1 µg/lane) were subjected to SDS-PAGE, followed by immunoblot analysis with anti-CL4 antibody. The lysate of CL4-expressing L (CL4/L) cells was used as a positive control. B, C) Interaction of a CL4 binder with CL4-BV. Immunotubes were coated with the wild-BV or CL4-BV, and C-CPE (B) or mutated C-CPE (C) was added to the BV-coated immunotubes at the indicated concentration. C-CPE bound to the BV-coated tubes was detected by ELISA with an anti-his-tag antibody.
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We analyzed the sequences of the CL4-BV-bound phages and got novel CL4-binder candidates with amino acid sequences that differed from the wild-type sequence (Table 2). To investigate their CL4-binding, we prepared the recombinant proteins of the binders and investigated their interaction with CL4 by ELISA with CL-BVs. As shown in Fig. 4A, the novel C-CPE derivatives had affinity to CL4 but not CL1. Next, we investigated whether the novel CL4 binders modulate TJ barrier in Caco-2 monolayer cell sheets, a popular model for the evaluation of TJ barriers [25].

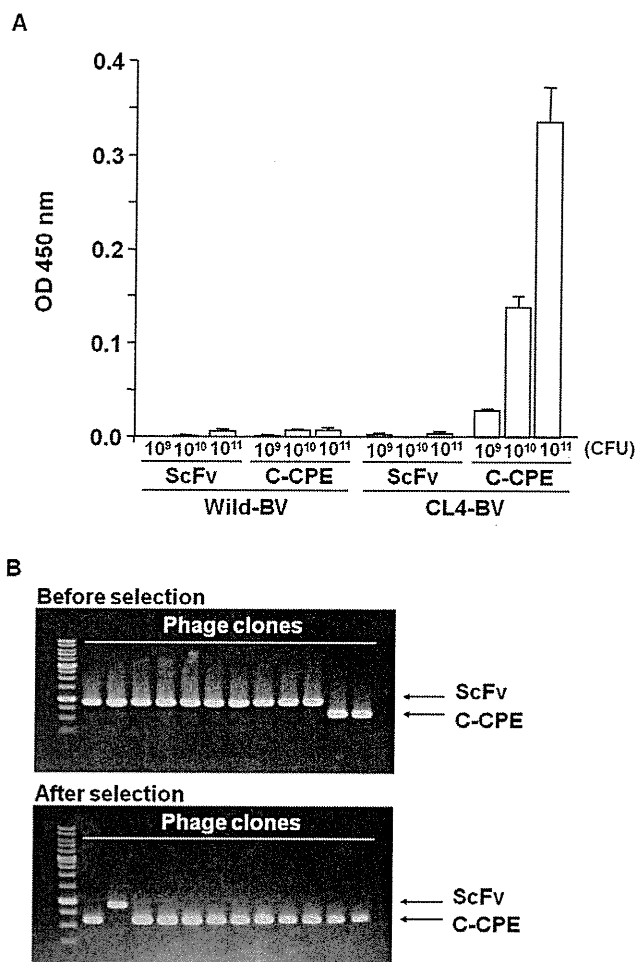

Figure 2. Selection of C-CPE-displaying phage by using the CL4-BV system. A) Interaction of C-CPE-displaying phage with CL4-BV. Wild-BV or CL4-BV was coated on an immunoplate, and then scFv-displaying phage or C-CPE-displaying phage was added to the BV-coated immunoplate at the indicated concentrations. The BV-bound phages were detected by ELISA with anti-M13 antibody as described in Materials and methods. Data are representative of two independent experiments. Data are means ± SD (n = 3). B) Enrichment of C-CPE-displaying phage by the BV system. A mixture of scFv-phage and C-CPE-phage (mixing ratio of scFv-phage to C-CPE-phage = 2:10) was incubated with a CL4-BV-coated immunotube, and the bound phages were recovered. Each phage clone was identified by PCR amplification, followed by agarose gel electrophoresis. Upper and lower pictures are before and after the selection, respectively. The putative sizes of the PCR products are 856 and 523 bp in scFv and C-CPE, respectively. The data are representative of two independent experiments.
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Treatment of the cells with C-CPE resulted in decreased transepithelial electrical resistance (TEER) values, a marker of TJ integrity, and the TEER values increased after removal of C-CPE. The C-CPE derivatives (clones 1–5) had TJ-modulating activity similar to that of C-CPE (Fig. 4B).

Discussion

CL is a promising target for pharmaceutical therapy. However, CL has low antigenicity, and there has been little success in the preparation of monoclonal antibody against the extracellular loop region of CL. The three-dimensional structure of CL has never been determined, so it is impossible to perform a theoretical design

Table 1. C-CPE phage library.

	304	305	307	309	313	318
C-CPE	S	S	S	N	S	K
Clone 1	V	T	C	V	N	K
2	C	P	A	H	L	T
3	A	G	G	V	P	P
4	R	G	H	L	E	H
5	A	A	P	S	R	Q
6	P	A	P	D	P	A
7	C	T	T	T	N	K
8	H	P	S	P	G	H
9	R	G	G	R	N	R
10	A	P	S	T	Q	P
11	V	L	G	N	M	R
12	P	P	A	T	F	R
13	G	D	C	S	N	L
14	F	R	V	F	R	N
15	S	Q	Q	W	T	T
16	S	R	L	E	W	Q
17	K	R	E	R	Q	S

Phage clones were randomly picked up from the C-CPE phage library, and the amino acids sequences of C-CPE mutant were analyzed.
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of a CL binder based on the structural information. In the present study, we developed a novel screening system for CL binders by using a BV system and a C-CPE phage display library, and we used this system to identify novel CL4 binders.

In ligand screening, the preparation of a receptor for the ligand is very critical. Membrane proteins are especially difficult to prepare as recombinant protein with an intact structure. Functional membrane proteins such as cell-surface proteins are heterologously expressed on BV in their native forms [19–21]. Interactions between membrane proteins can be detected by using receptor-displaying and ligand-displaying BV [21]. In the present report, we found that CL4-BV interacts with a CL4 binder, C-CPE, but it does not interact with C-CPE303 that lacks the CL4-binding residues of C-CPE. The CL4-binding site of C-CPE corresponds to that of CPE; so, the second extracellular loop of CL appears to be the C-CPE-binding site [23,26]. These findings indicate that CL4 displayed on BV may have native form. We anticipate that CL-BV will be useful for the preparation of CL binders, such as peptides and antibodies.

To the best of our knowledge, the preparation of CL binder has been performed by only four groups. Offner et al. prepared polyclonal antibodies against extracellular domains of CL3 and CL4 [27], Ling et al. screened peptide types of CL4 binder by using a 12-mer peptide phage display library and CL4-expressing cells [28], Suzuki et al. generated a monoclonal antibody against the second extracellular loop of CL4 from mice immunized with a human pancreatic cancer cell line [29] and Romani et al. screened scFv against CL3 by using a human antibody phage display library [30]. However, the CL modulators have never been developed; thus, C-CPE is the only known CL4 modulator [12]. In the present study, we prepared a C-CPE phage library containing C-CPE mutants in which each of the 6 functional amino acids was randomly replaced with an amino acid, and we isolated CL4 binders by using CL4-BV as a screening ligand. Interestingly, all of

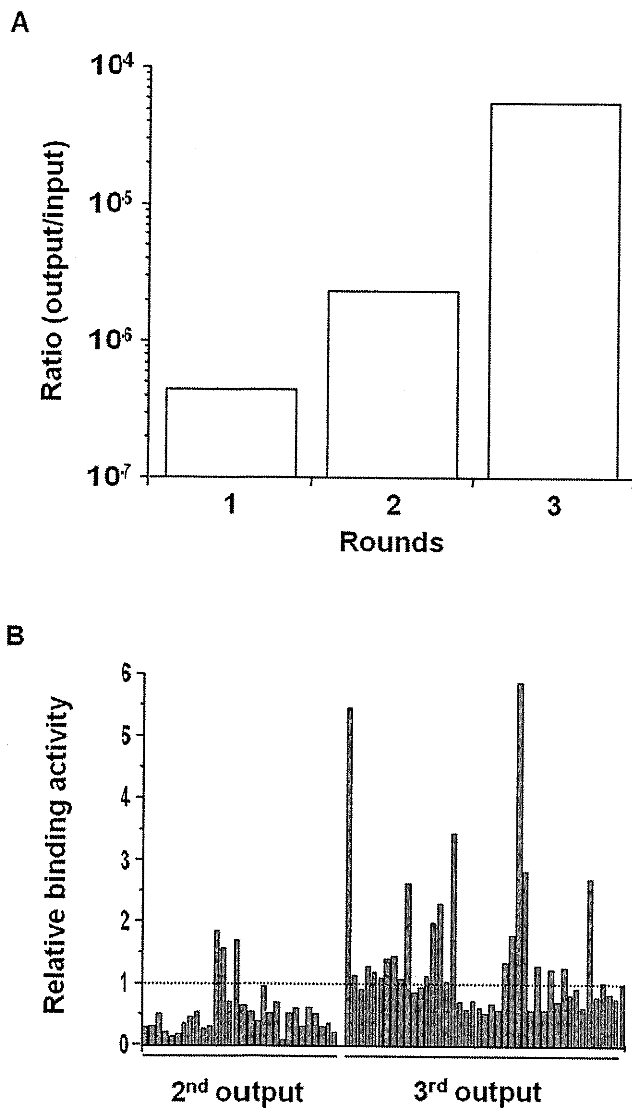

Figure 3. Screening of a novel CL4 binder. A) Enrichment of phages with affinity to CL4-BV. CL4-BVs coated on immunotubes were incubated with the C-CPE-derivative phage library at 1.6×10^{12} CFU titer (1st input phage). The phages bound to CL4-BV were recovered (1st output phage). The CL4-BV-binding phages were subjected to two additional cycles of the incubation and wash step, resulting in 2nd, 3rd output phage. The ratio of output phage to input phage titers was calculated. B) Monoclonal analysis of C-CPE-derivative phage. CL4-BV-bound phage clones were isolated from the 2nd and 3rd output phages, and the interaction of the monoclonal phage with CL4-BV was examined by ELISA with anti-M13 antibody as described in Materials and methods. Data are expressed as relative binding to that of C-CPE-phage indicated by the most right column.
doi:10.1371/journal.pone.0016611.g003

the CL4 binders modulated TJ barriers. We are investigating why the substitution of the amino acids with the other amino acids modulated CL4. These findings indicate that a BV screening system with a C-CPE library may be a powerful method to develop CL modulators.

The CL family forms various types of TJ barriers through combinations of its more than 20 members in homophilic/heterophilic CL strands [31,32]. Intercellular proteins ZO-1 and ZO-2 determine the localization of CL strands [33]. If a screening system to reconstitute heterogeneous CL strands with ZO-1 and/or ZO-2 is developed, then useful and effective CL modulators can be identified. In this point, the BV system has extremely superior features. G protein and G protein-coupled receptors have been

Table 2. CL4-binding phages.

	304	305	307	309	313	318
C-CPE	S	S	S	N	S	K
Clone 1	R	V	S	A	R	R
2	R	S	V	A	R	K
3	G	D	G	R	T	R
4	S	A	P	R	S	A
5	R	S	L	K	S	K

The sequences of C-CPE mutant in the CL4-binding phages were analyzed.
doi:10.1371/journal.pone.0016611.t002

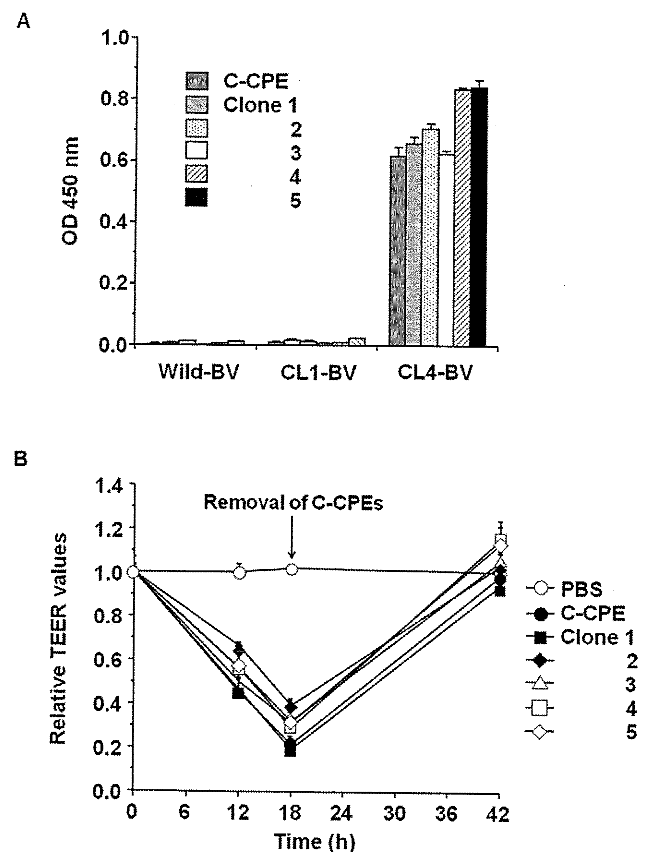

Figure 4. Isolation of a novel CL4 modulator. A) Interaction of the C-CPE derivatives with CL4. C-CPE derivatives were prepared as his-tagged recombinant proteins. The C-CPE derivatives (0.02 µg) were added to CL-BV-coated immunoplates, followed by detection of the C-CPE derivatives bound to CL-BV. Data are means ± SD (n = 4). B) Modulation of tight junction-barriers. Caco-2 cells were cultured on Transwell™ chambers. When TEER values reach a plateau, the cells were treated with C-CPE or C-CPE derivatives at the indicated concentrations. After 18 h of exposure to the C-CPEs, the cells were washed with medium to remove C-CPEs, and then the cells were cultured for an additional 24 h. Changes in TEER values were monitored during the C-CPEs treatment. Relative TEER values were calculated as the ratio of TEER values at 0 h. Data are representative of two independent experiments. The data are means ± SD (n = 4).
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functionally reconstituted in BV [20,34], and functional γ-secretase complexes have also been reconstituted on BV [35]. In the near future, the reconstituted CL system on BV will be developed and used for the screening of CL binders and modulators, hopefully leading to breakthroughs in pharmaceutical therapies that target CLs.

Materials and Methods

Recombinant BV construction and Sf9 cell culture

Recombinant BV was prepared by using the Bac-to-Bac expression system, according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD). Briefly, mouse CL1 and CL4 cDNA (kind gifts from Dr. M Furuse, Kobe University, Japan) were inserted into pFastBac1, and the resulting plasmids were transduced into DH10Bac *E. Coli* cells. Recombinant bacmid DNA was extracted from the cells. Sf9 cells were transduced with the bacmid coding CL, and the recombinant BV was recovered by centrifugation of the conditioned medium [36].

Preparation of the BV fractions

Sf9 cells (2×10^6 cells) were infected with recombinant BV at a multiplicity of infection of 5. Seventy-two hours after infection, the BV fraction was recovered from the culture supernatant of infected Sf9 cells by centrifugation. The pellets of the BV fraction were resuspended in Tris-buffered saline (TBS) containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and then stored at 4°C until use. The expression of CL1 and CL4 in the BV was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with anti-CL antibodies (Zymed Laboratory, South San Francisco, CA).

Preparation of mutant C-CPE library

C-CPE fragments in which the functional amino acids (S304, S305, S307, N309, S313 and K318) [24] were randomly mutated were prepared by polymerase chain reaction (PCR) with pET-H_{10}PER as a template, a forward primer (5′-catgccatggccgatatagaaaaagaaatccttgatt-tagctgctg-3′, Nco I site is underlined) and a reverse primer (5′-ttttccttttgcggccgcaaa*snn*ttgaaataatat*snn*ataagggta*snn*tcc*snn*ata*snnsnn*at-tagcttt-3′, Not I site is underlined, and the randomly mutated amino acids are in italics). The PCR fragments were inserted into a pY03 phagemid at the NcoI/NotI sites [22]. The resultant phagemid containing the C-CPE mutant library was transduced into *E. coli* TG1 cells, and then the cells were stored at −80°C.

Preparation of phage

TG1 cells containing phagemid coding a scFv, C-CPE, C-CPE mutant or C-CPE mutant library were culture in 2YT medium containing 2% glucose and ampicillin. When the cells grew to a growing phage, M13K07 helper phages (Invitrogen) were added, and the medium was changed into 2YT medium containing ampicillin and kanamycin. After an additional 6 h of culture, the phages in the conditioned medium were precipitated with polyethylene glycol. The phages were suspended in phosphate-buffered saline (PBS) and stored at 4°C until use.

ELISA

Wild-BVs or CL-BVs (0.5 µg/well) were adsorbed onto an immunoplate (Greiner Bio-One, Frickenhausen, Germany). The wells were washed with PBS and blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). C-CPEs or phages were incubated in the immunoplate, and the BV-bound C-CPEs or phages were detected by using anti-his-tag antibody (Novagen, Darmstadt, Germany) or anti-M13 antibody (Amersham-Pharmacia Biotech, Uppsala, Sweden), respectively, horseradish peroxidase-labelled secondary antibody and TMB peroxidase substrate (Nacalai Tesque, Kyoto, Japan). The immunoreactive C-CPEs or phages were quantified by the measurement of absorbance at 450 nm. In the screening of phages, the data were normalized by the amounts of phages, which were quantified by ELISA for the FLAG-tag contained in the coat protein.

Selection of phage by using BV

A total of 0.5 µg of BV was adsorbed onto an immunotube (Nunc, Roskilde, Denmark). The tube was washed with PBS and blocked with TBS containing 4.0% BlockAce. The BV-coated tubes were incubated with mixture of phages, and then the tubes were washed 15 times with PBS and 15 times with PBS containing 0.05% Tween 20. The phages bound to the tube were eluted with 100 mM HCl. TG1 cells were infected with the eluted phages, and phages were prepared as described above. The resulting phages were subjected to repeated selection by using the BV-coated immunotubes.

Identification of a phage clone

To identify an isolated phage clone, we performed PCR or sequencing analysis. We amplified the inserted fragment into the phagemid by PCR using forward primer 5′-caggaaacagctatgac-3′ and reverse primer 5′-gtaaatgaattttctgtatgagg-3′. The resultant PCR products were subjected to agarose gel electrophoresis followed by staining with ethidium bromide. We performed a sequence analysis with primer 5′-gtaaatgaattttctgtatgagg-3′.

Measurement of phage titer

To quantify the concentration of phages, we measured the titer (colony formation unit (CFU)/ml) of the phage solution. Briefly, the phage solution was diluted to 10^{-5}–10^{-10} with PBS. The diluted solution was seeded onto Petrifilm™ (Tech-Jam, Osaka, Japan). After 24 h of incubation, the colonies were counted, and the titer was calculated.

Purification of C-CPE mutants

C-CPE and C-CPE303, in which the CL-4 binding region of C-CPE was deleted, were prepared as described previously [13]. To prepare plasmid containing C-CPE mutants, the C-CPE mutant fragment was PCR-amplified by using phagemids coding C-CPE mutants as a template. The resulting PCR fragment was inserted into pET16b, and the sequence was confirmed. The plasmids were transduced into *E. coli* strain BL21 (DE3), and production of mutant C-CPEs 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 phenylmethanesulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol) that was supplemented with 8 M urea when necessary. The lysates were applied to HiTrap™ Chelating HP (GE Healthcare, Buckinghamshire, UK), and mutant C-CPEs were eluted with buffer A containing 100–400 mM imidazole. The buffer was exchanged with PBS by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80°C until use. Purification of the mutant C-CPEs was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-his-tag antibody (Novagen). Protein was quantified by using a BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, Rockford, IL).

TEER assay

Caco-2 cells were seeded in Transwell^{TM} chambers (Corning, NY) at a subconfluent density. The TEER of the Caco-2 monolayer cell sheets on the chamber was monitored by using a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). When TEER values reached a plateau, indicating that TJs were well-developed in the cell sheets, the Caco-2 monolayers were treated with C-CPE or C-CPE mutants on the basal side of the chamber. Changes in TEER values were monitored. The TEER values were normalized by the area of the Caco-2 monolayer, and the TEER value of a blank Transwell^{TM} chamber (background) was subtracted.

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Author Contributions

Conceived and designed the experiments: MK TS TH KY. Performed the experiments: HK AT MK YS TY TS. Analyzed the data: HK AT MK KY. Contributed reagents/materials/analysis tools: HK AK TS TH. Wrote the manuscript: HK MK TY.

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A toxicological evaluation of a claudin modulator, the C-terminal fragment of *Clostridium perfringens* enterotoxin, in mice

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Tight junctions (TJs) maintain cellular polarity between the apical and basolateral region of epithelial cells. Claudin, a tetra-transmembrane protein, plays a pivotal role in the barrier function of TJs. We previously found that a claudin modulator, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), may be a promising candidate for improving the mucosal absorption of drugs. C-CPE is a fragment of enterotoxin, and putative CPE claudin receptors are highly expressed in liver and kidney. The safety and antigenicity of C-CPE must be evaluated for future clinical application. Therefore, we evaluated whether C-CPE administration in mice leads to tissue injury or production of antibodies. Intravenous administration of C-CPE at 5 mg/kg, which is a more than 25-fold higher dose than that used in a murine mucosal absorption model, did not increase biochemical markers of liver and kidney injury even after 11 injections once a week. Nasal C-CPE administration (2 mg/kg) once a week for 11 administrations also did not increase these biochemical markers, but 6 administrations of C-CPE resulted in elevation of C-CPE-specific serum IgG. These results indicate that development of a less antigenic claudin modulator will be essential for future clinical application of a C-CPE-based mucosal absorption enhancer.

1. Introduction

The use of biologics, such as antibodies, peptides, and nucleic acids, in new drugs is becoming increasingly prominent. Biologics are biodegradable and poorly absorbed in the mucosa, and therefore they are often employed as injectable drugs. The development of a non-invasive system for delivery of drugs across the mucosal epithelium would improve quality of life and patient compliance. Since orally administered drugs can be degraded by digestive enzymes and first pass effects in the liver, developing ways to administer drugs through nasal and pulmonary transmucosal absorption has a high priority. However, passing biologics across the mucosal epithelium is extremely difficult because the mucosa's primary function is as a physical and biological barrier preventing the entry of pathogens and toxic substances into the body.

Epithelial cell sheets develop intercellular junctions to prevent the free movement of solutes between sheets. Adjacent epithelial cells adhere to one another via tight junctions (TJ), adherent junctions, and gap junctions. Among these, the TJ plays a key role in sealing the intercellular space and preventing leakage of solutes. Modulation of the TJ barrier has proven to be a promising strategy for enhancement of mucosal drug absorption. Tight junction modulators, such as surfactants, chelators, and nitric oxide donors, have been investigated as potential absorption enhancers since the 1960 s (Aungst 2000; Citi 1992; Engel and Riggi 1969; Tomita et al. 1996).

The detection and development of absorption-enhancers focuses on modulating activity of the TJ barrier. Such enhancers are called "the first generation TJ modulators" (Kondoh et al. 2008). The identification of claudin, a structural and functional TJ component, provided new insight into absorption-enhancers, and led to a TJ-components-based strategy for enhancer development, the second generation TJ modulators. Claudins are ~23 kDa proteins bearing tetra-transmembrane domains and comprise a family of more than 20 members (Furuse and Tsukita 2006). The expression profiles and barrier function of the various claudin family members differ among tissues. For instance, claudins-1 and -5 are critical for epidermal barrier and blood-brain-barrier functions, respectively (Furuse et al. 2002; Nitta et al. 2003). Modulation of the claudin barrier has been proposed as a novel strategy for absorption enhancement (Furuse et al. 1998; Tsukita and Furuse 1998).

Clostridium perfringens enterotoxin (CPE) is a cause of food poisoning in humans (McClane and Chakrabarti 2004). A receptor of CPE is identical to claudin-3/4, and the C-terminal fragment of CPE (corresponding to amino acids 184–319) modulates the TJ barrier by its interaction with claudin-3/4 (Sonoda et al. 1999). We found that the claudin modulator the C-terminal fragment of CPE was 400-fold more potent at enhancing intestinal absorption than a clinically used absorption-enhancer, sodium caprate (Kondoh et al. 2005). However, the C-terminal fragment of CPE did not enhance intestinal absorption of a peptide drug when co-administered (Uchida et al. 2010). The N-terminal truncated fragment (C-CPE), comprising amino acids 194–319, did enhance intestinal, nasal, and pulmonary absorption of a biologically active peptide (Uchida et al. 2010). Thus, C-CPE may be a promising enhancer of mucosal drug absorp-

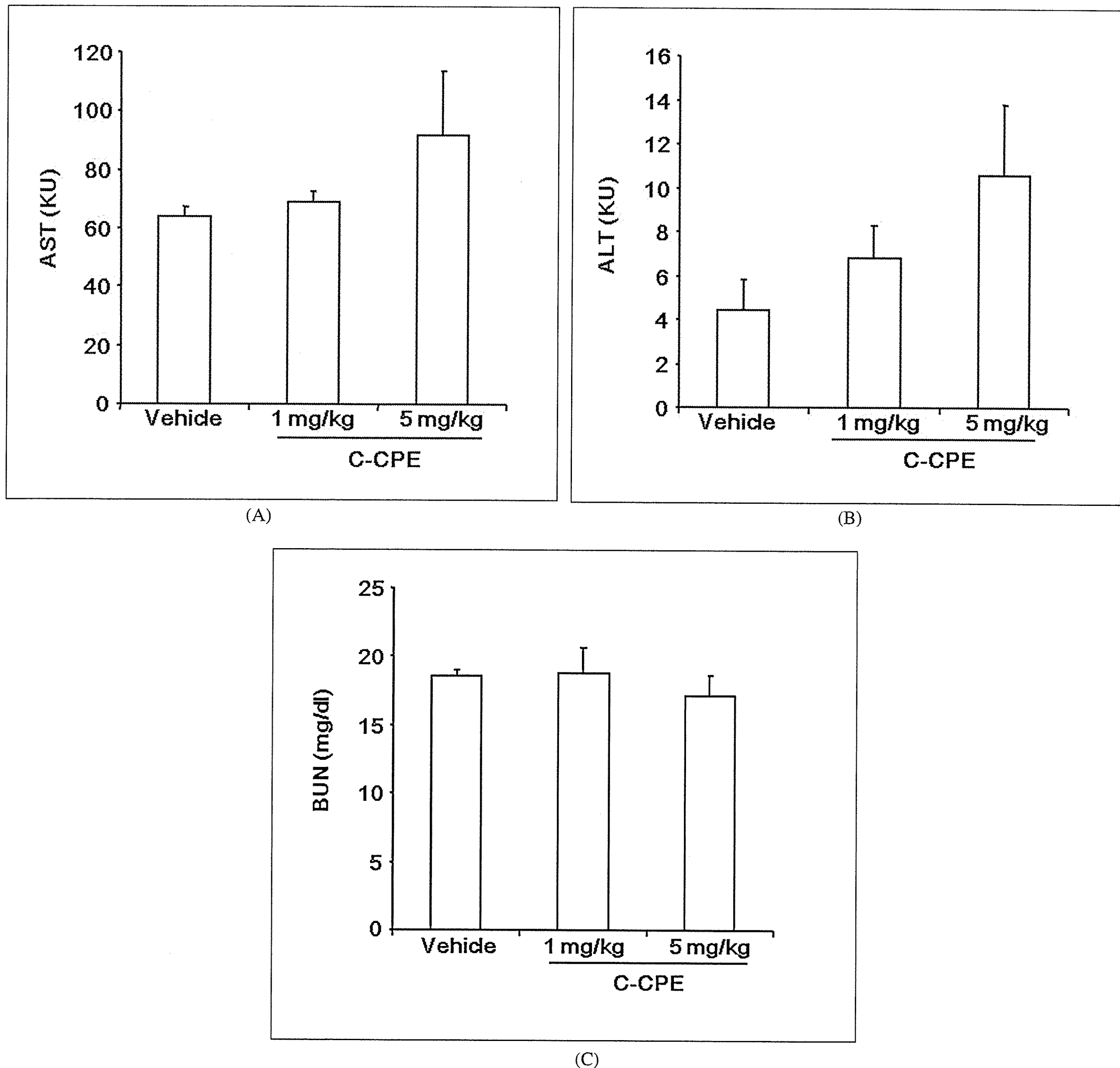


Fig. 1: Effect of systemic injection of C-CPE on biochemical markers for liver and kidney injury. Mice received intravenous injections of C-CPE at 0, 1, or 5 mg/kg once a week for 11 weeks. Blood was recovered 24 h after the last administration of C-CPE. Serum AST (A), ALT (B) and BUN (C) were measured using a commercially available kit as described in the Expreimental section. Data are presented as mean ± SEM (n = 3 or 5)

tion. Although data on safety and antigenicity is critical for any future clinical application of C-CPE, its potential side effects have not been investigated.

In the present study, we investigated the effect of C-CPE administration on liver and kidney tissues in which claudin-3/4 is expressed, as well as studied induction of anti-C-CPE antibodies.

2. Investigations, results and discussion

Administration of C-CPE enhanced the mucosal absorption of dextran with a molecular weight of 20 kDa, indicating that C-CPE (13 kDa) might enter into the systemic flow from the mucosal membrane with drugs (Kondoh et al. 2005). C-CPE constitutes the receptor-binding domain of CPE, and binds to claudin-3 and claudin-4 (Fujita et al. 2000; Katahira et al. 1997). Since claudins-3 and -4 are highly expressed in the liver and kidney (Morita et al. 1999), we evaluated the effect of C-CPE on these tissues. To investigate the potential effects of C-CPE on liver and kidney, we systemically injected C-CPE into mice once a week for 11 weeks and measured biochemical markers of liver (AST and ALT) and kidney (BUN) injury 24 h after the last injection. As shown in Figs. 1A, 1B and 1C, intravenous administration of C-CPE did not affect serum AST, ALT and BUN levels, even at a dose as high as 5 mg/kg. C-CPE was mucosally administered at 0.02–0.4 mg/kg (Uchida et al. 2010). Therefore, even if all C-CPE was absorbed, no side effects in liver or kidney are likely to occur.

Since C-CPE is a polypeptide, its antigenicity could interfere with its clinical use. We therefore investigated whether repeated mucosal administration of C-CPE activates serum C-CPE-specific IgG responses. Mice were surgically operated upon in jejunal and pulmonary absorption studies, and consequently could not be repeatedly treated with C-CPE. Therefore, to investigate the antigenicity of C-CPE following mucosal administration, C-CPE was intranasally administered to mice once a week for 10 weeks. Serum IgG production was monitored every week. C-CPE treatment did not increase C-CPE-specific serum IgG after 4 administrations of C-CPE at 2 mg/kg. However, 6 administrations of C-CPE did cause production of C-CPE-

specific serum IgG (Fig. 2). A dose of 1 mg/kg is equal to that used in a previously published study on mucosal absorption (Uchida et al. 2010). Repeated mucosal administration of C-CPE in our study at twice this dose (2.0 mg/kg) did not increase serum AST, ALT and BUN levels (Figs. 3A 3B and 3C). These findings indicate that while C-CPE does not cause tissue damage at clinically relevant doses, it may be limited in its clinical applications as a mucosal absorption enhancer only by its antigenicity.

There are two potential directions for clinical applications of claudin modulators. The first is preparation of a claudin modulator based on C-CPE. An antigenic determinant assay of CPE revealed that the C-terminal fragment corresponding to amino acids 286–305 was immunogenic (Sugii 1994). Mutating the antigenic domain while maintaining its claudin-binding activity would contribute to development of a low antigenic claudin modulator. In general, smaller peptides are less antigenic. The C-terminal fragment corresponding to amino acids 290–319 constituted the receptor-binding domain of CPE (Hanna et al. 1991). Preparation of a claudin-modulating peptide with low antigenicity and high claudin-modulating activity using this 30 amino acid fragment may lead to a claudin modulator useful as an enhancer of drug absorption.

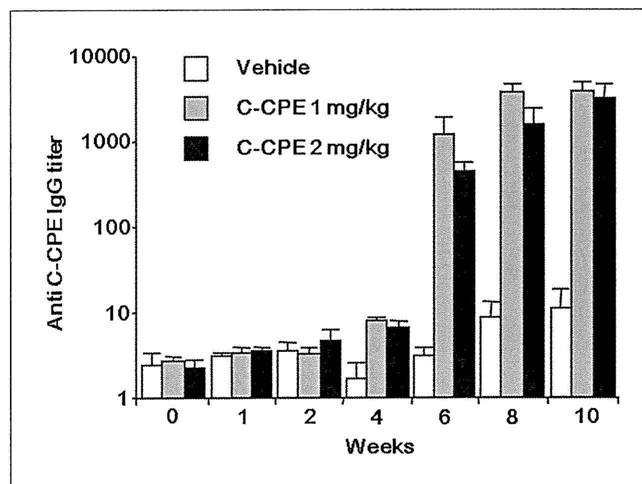
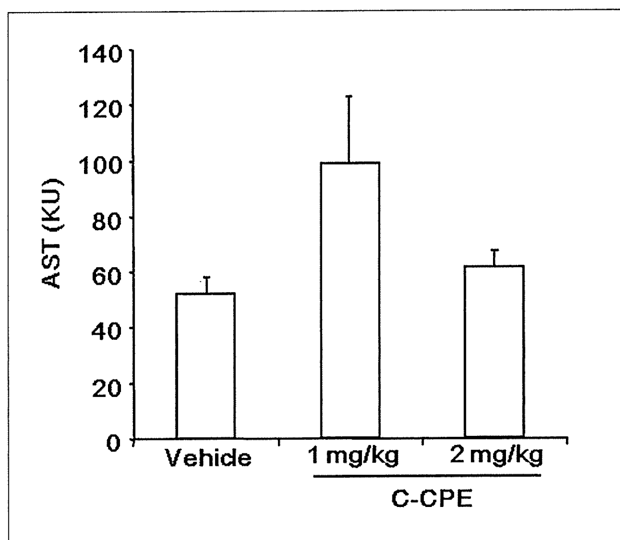
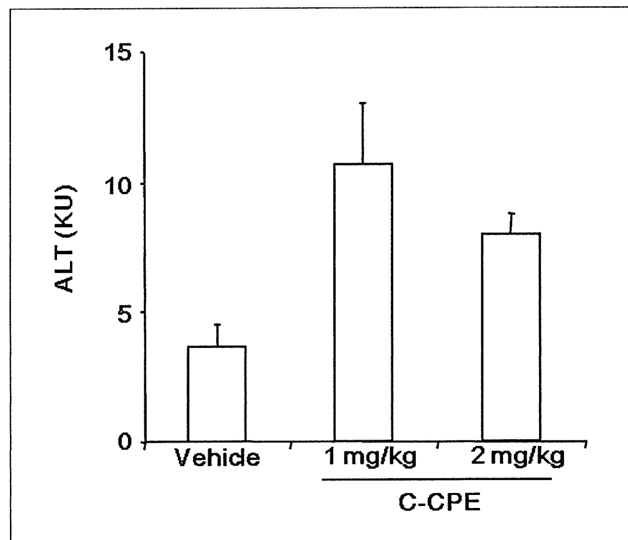


Fig. 2: Effect of mucosal administration of C-CPE on production of anti-C-CPE IgG. Mice received nasal injections of C-CPE at 0, 1, or 2 mg/kg once a week for 10 weeks. Blood was collected each week and serum C-CPE specific IgG levels were measured as described in Materials and methods. Data are presented as means ± SEM (n = 4 or 5)



(A)



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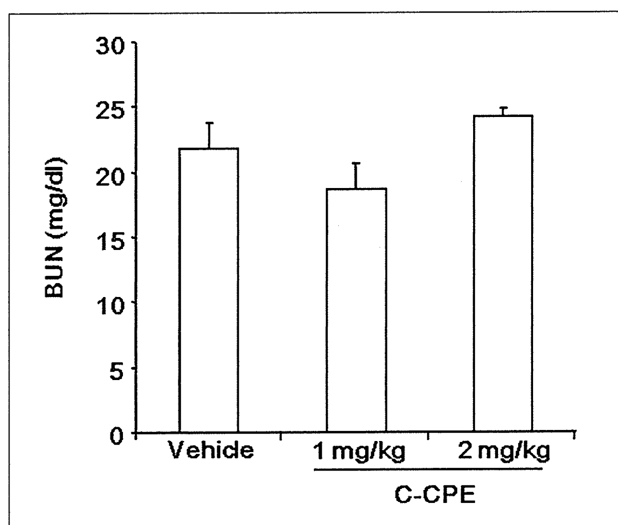

(C)

Fig. 3: Effect of repeated mucosal administration of C-CPE on biochemical markers of liver and kidney injury. Mice were nasally administered with C-CPE at 0, 1, or 2 mg/kg once a week for 11 weeks. Seven days after the last administration, blood was recovered, and serum AST (A), ALT (B) and BUN (C) were measured using a commercially available kit. Data are presented as mean ± SEM (n = 4 or 5)

The second potential direction for clinical applications of claudin modulators lies in the preparation of a human antibody to modulate the claudin-barrier. Claudin is characterized by low antigenicity, and it is therefore difficult to prepare an antibody to bind its extracellular domain (Evans et al. 2007). Recently, Romani et al. successfully prepared a human single-chain antibody to claudin-3, and Suzuki et al. also prepared a monoclonal antibody against claudin-4 (Romani et al. 2009; Suzuki et al. 2009). Development of a humanized antibody against claudin will contribute greatly to the clinical applications of claudin modulators.

In summary, we found that C-CPE administration does not result in significant tissue injury in mice. However, our findings also suggest that discovering a means of reducing C-CPE's antigenicity is critical for continued development of a C-CPE-based claudin modulator useful as an enhancer of drug absorption.

3. Experimentals

3.1. *Animals*

BALB/c female mice (6 wk) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), and were housed in an environmentally controlled room at 23 ± 1.5 °C with a 12-h light/12-h dark cycle. The mice had free access to water and commercial chow (Type MF, Oriental Yeast, Tokyo, Japan). Experimental protocols involving mice were performed according to the ethics guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

3.2. *Preparation of C-CPE*

C-CPE was prepared as described previously (Uchida et al. 2010). Briefly, pET16b vector plasmids coding the C-terminal fragment of CPE (amino acids 194–319) were transduced into *E. coli* BL21 (DE3), and protein expression was stimulated by addition of isopropyl-1-thio-β-D-galactoside. Cell lysates were applied to HisTrap^{TM} Chelating HP columns (GE Healthcare, Buckinghamshire, UK), and C-CPE was eluted with imidazole. The solvent was exchanged with phosphate-buffered saline by gel-filtration, and the purified proteins were stored at −80 °C until use. Purification of the proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by staining with Coomassie Brilliant Blue. C-CPE was quantified by using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL) using bovine serum albumin as a standard.

3.3. *Biochemical assay*

C-CPE was administered to mice intravenously or nasally once a week for 11 weeks, and blood was collected from the mice by cardiac puncture one day after the last administration. Serum aspartate aminotransferase (AST), alanin aminotransferase (ALT) levels and blood urea nitrogen (BUN) were measured using commercially available Transaminase-CII and Blood Urea Nitrogen-B Test (WAKO Pure Chemical, Osaka, Japan) kits, respectively.

3.4. *C-CPE-specific antibody production*

C-CPE was administered to mice intravenously or nasally once a week. Serum was collected 7 days after each administration of C-CPE. The titers of C-CPE-specific antibody in serum were determined using an enzyme-linked immunosorbent assay. Briefly, an immunoplate was coated with C-CPE (1 μg/well in a 96-well plate). Ten-fold serial dilutions of samples were added to the wells, followed by reaction with horseradish peroxidase-conjugated anti-mouse IgG. The presence of C-CPE-specific antibodies was determined 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.

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Adenovirus vector-mediated assay system for hepatitis C virus replication

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ABSTRACT

The efficient delivery of the hepatitis C virus (HCV) RNA subgenomic replicon into cells is useful for basic and pharmaceutical studies. The adenovirus (Ad) vector is a convenient and efficient tool for the transduction of foreign genes into cells *in vitro* and *in vivo*. However, an Ad vector expressing the HCV replicon has never been developed. In the present study, we developed Ad vector containing an RNA polymerase (pol) I-dependent expression cassette and a tetracycline-controllable RNA pol I-dependent expression system. We prepared a hybrid promoter from the tetracycline-responsive element and the RNA pol I promoter. Ad vector particles coding the hybrid promoter-driven HCV replicon could be amplified, and interferon, an inhibitor of HCV replication, reduced HCV replication in cells transduced with the Ad vector coding HCV replicon. This is the first report of the development of an Ad vector-mediated HCV replicon system.

INTRODUCTION

Hepatitis C virus (HCV) is a member of *Flaviviridae* that contains a 9.6-kb positive-sense RNA genome. A total of 170-million people worldwide are infected with HCV, leading to chronic hepatic inflammation, hepatic fibrosis, hepatic cirrhosis and hepatocellular carcinoma (1). Chronic infection with HCV is a major cause of hepatocellular carcinoma (1). Interferon (IFN) therapy is the gold standard method for HCV patients, but it is effective in only 50% of patients and its use has been limited because of severe side effects (2–4). Additional pharmaceutical therapies are needed to overcome HCV. However, the tropism of HCV is limited to chimpanzees and humans, and the mechanism of HCV infection and replication is not fully understood. The HCV genome encodes a polyprotein precursor of about 3000 amino acids that is cleaved into at least 10 proteins: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (5). An HCV subgenome replicon (called HCV replicon in the present study) consisting of a reporter gene and HCV NS genes has allowed various studies of HCV replication and the development of anti-HCV agents (6–8). The delivery of the HCV genome or HCV replicon is a powerful tool for basic and pharmaceutical research, and the transduction of *in vitro* translated HCV RNA genome is often performed by electroporation. However, a convenient and efficient method to transfer the 9.6-kb HCV RNA genome or the 8–9-kb HCV replicon has never been fully developed.

Transcribed RNAs are classified into rRNAs, mRNAs and short RNAs (tRNAs) in mammalian cells. RNA polymerases differ among the transcribed RNA species: RNA polymerase (pol) I for rRNAs, RNA pol II for mRNA and RNA pol III for short RNAs. RNA pol I transcribes RNA without a 5′-cap structure or a 3′-poly-A tail, and a plasmid vector encoding RNA pol I promoter and terminator has been applied to the development of RNA virus-expression system. For instance, influenza viruses, arenavirus and uukuniemi viruses are generated using RNA pol I-driven expression plasmid vectors coding each segment of negative-sense RNA (9–12). Recombinant adenovirus (Ad) vectors have been widely used to deliver foreign genes to a variety of cell types and tissues *in vitro* and *in vivo* in basic research and clinical therapy. Ad vector can be easily prepared, grown to a high titer, and used to efficiently transfer genes into dividing and non-dividing cells. Furthermore, several types of Ad vectors have been developed to expand their tropism and to increase the size of encoded genes (13,14). Ad vector encoding RNA pol I-driven expression of influenza virus

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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RNA has been developed for the generation of vaccine seed strains and for basic influenza virus studies (15). These findings indicate that the RNA pol I Ad vector system can be a promising tool for basic and pharmaceutical studies on HCV. However, the development of an RNA pol I-driven vector system expressing the HCV RNA genome has never been reported.

In the present study, we developed an RNA pol I-driven vector system to monitor HCV replication using an HCV replicon in which structural genes were replaced by the luciferase gene. We prepared an Ad vector containing a tetracycline (tet)-regulated RNA pol I-expression cassette consisting of an RNA pol I-driven responsive vector and a *trans*-activator vector, and we successfully developed an Ad vector-mediated HCV replication system.

MATERIALS AND METHODS

Cell culture

Huh7.5.1 1bFeo [genotype 1b HCV replicon cell line, (8)] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and G418 (Nacalai Tesque, Kyoto, Japan) at 500 µg/ml. Huh7 and 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. The cells were maintained in a 5% CO_2 atmosphere at 37°C.

Preparation of RNA pol I-driven plasmid vectors

An RNA pol I expression-cassette was subcloned as follows: pHH21 (kindly provided by Dr Kawaoka, Tokyo, Japan) containing RNA pol I expression cassette was digested with *Afl*III, blunted by the Klenow fragment of DNA polymerase, ligated with *Eco*RI linker and digested with *Eco*RI/*Nhe*I, resulting in a fragment of the RNA pol I expression-cassette. The RNA pol I cassette was inserted into the *Eco*RI-*Xba*I site of pHM5 (16), generating pPol I. A fusion gene of enhanced green fluorescent protein and firefly luciferase (EGFPLuc, Clontech, Mountain View, CA, USA) was inserted into pPol I, resulting in pP_IWT-EL.

The subgenomic HCV sequence and the replication-incompetent subgenomic HCV sequence deleting GDD motif (MLVNGDDLVV) in NS5B were amplified by polymerase chain reaction (PCR) using pRepFeo as a template (8). The PCR fragments were inserted into pPol I, generating pPol I-1bFeo and pPol I-1bFeoΔGDD. The Feo fragment in pPol I-1bFeo or pPol I-1bFeoΔGDD was replaced with firefly luciferase, generating pPol I-HCV or pPol I-ΔGDD coding firefly luciferase reporter, HCV NS3, NS4A, NS5A and NS5B or mutated NS5B, respectively. A plasmid expressing β-galactosidase, pCMVβ, was purchased from Marker Gene Inc. (Eugene, OR, USA).

Preparation of tet-controllable RNA pol I-driven plasmid vectors

To develop the tet-controllable RNA pol I promoter expression system, the minimal cytomegalovirus promoter was replaced by fragments of RNA pol I promoters (from −235 to −1, from −311 to −1 or from −412 to −1) in pHM5-TREL2 (17), generating pP_I235, pP_I311 or pP_I412. These RNA pol I plasmid vectors were used for optimization of the tetracycline responsive element (TRE)/RNA pol I chimeric promoter. pHM5-tTA, pHM5-rtTA and pHM5-TREL2 were used in tet-regulated experiments (17).

Preparation of Ad vector expressing HCV replicon

The HCV replicon fragments cloned from pPol I-HCV or pPol I-ΔGDD were inserted into pP_I235, and then the firefly luciferase was replaced by the renilla luciferase to form pP_I235-HCV or pP_I235-ΔGDD. Ad vectors were constructed by an improved *in vitro* ligation method (18). Briefly, pP_I235-EL, pP_I235-HCV and pP_I235-ΔGDD were digested with I-*Ceu*I and PI-*Sce*I, and then ligated with I-*Ceu*I/PI-*Sce*I-digested pAdHM4 and pAdHM36, respectively. The resulting plasmids were digested with *Pac*I and transfected into 293 cells with SuperFect (Qiagen, Valencia, CA, USA). AdP_I235-EL, AdP_I235-HCV and AdP_I235-ΔGDD were purified by $CsCl_2$ gradient centrifugation and dialyzed with a solution containing 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 10% glycerol. The multiplicity of infection (MOI) of Ad vectors was measured using an Adeno-X rapid titer kit (Clontech). Ad-tTA vectors were prepared as previously described (17).

Expression of plasmid-based HCV replicon

Huh7 cells were transfected with 0.8 µg of pPol I-HCV. After 24 h of incubation, the cells were lysed in LCβ (Toyo Ink, Tokyo, Japan). The cell lysates were frozen-thawed and centrifuged at 32 000 *g* for 5 min. The luciferase activity in the resulting supernatant was measured using a commercially available kit (PicaGene; Toyo Ink).

Inhibition assays of HCV replication in plasmid- or Ad-based RNA pol I HCV system

Huh7 cells were transfected with 0.8 µg of pPol I-HCV and 0.2 µg of pCMVβ or infected with AdP_I235-HCV (10 MOI) and Ad-tTA (50 MOI). After 2.5 or 1.5 h of transfection, the cells were treated with recombinant human interferon-α8 (IFN-α8) at the indicated concentration. After an additional 72 h of incubation, the cells were lysed in LCβ. Luciferase activity and β-galactosidase activity in the lysates was measured with PicaGene and a Luminescent β-gal Kit (Takara Bio Inc., Shiga, Japan), respectively. The cell viability was measured with a WST-8 kit according to the manufacturer's instruction (Nacalai Tesque).

Evaluation of tetracycline-controllable promoters in plasmid vector

Huh7 cells were co-transfected with 0.1 µg of reporter plasmid (pP_I235-EL, pP_I311-EL, pP_I412-EL or pP_IWT-EL), 0.8 µg of tet-responsive *trans*-activator plasmid (pHM5-rtTA in the tet-on system or pHM5-tTA in the tet-off system) and 0.1 µg of pCMVβ. After 2.5 h, the cells were treated with doxycycline (Dox) at the indicated

concentration for 48 h. Then, luciferase and β-galactosidase activities in the lysates were measured.

Expression of Ad vector containing tetracycline-controllable promoter system

Huh7 cells were transfected with a reporter Ad vector (AdP_I235-EL or AdP_I235-HCV at MOI of 5 or 10) and a *trans*-activator vector (Ad-tTA at MOI of 10 and 50). After an additional 48 h of incubation, luciferase activity in the cell lysates was measured.

Western blotting

Huh7 cells were co-infected with AdP_I235-HCV at 10 MOI and Ad-tTA at 50 MOI. The cells were lysed in RIPA buffer [50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA] containing a cocktail of protease inhibitors (Sigma, St Louis, MO, USA). The cell lysates (30 μg of protein) were subjected to sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE), followed by western blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking in 5% skim milk, the filter was incubated with mouse anti-NS5A (Meridian Life Science, Sacao, ME, USA) or anti-β-actin Ab (Sigma). Then, the peroxidase-labeled secondary antibodies were added. The immunoreactive bands were visualized by chemilumines-cence reagents (GE Healthcare, Buckinghamshire, UK).

Evaluation of NS5B-dependent replication

Huh7 cells were transfected 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, luciferase activities in the lysates were measured.

Detection of a fragment of the HCV negative strand RNA

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. The total RNAs were purified with High Pure RNA Isolation kit (Roche, Mannheim, Germany). The RNAs were reverse-transcribed to cDNA using a commer-cial available kit [TaKaRa RNA PCR Kit (AMV) Ver. 3.0] and a primer for the HCV negative strand RNA (5′-GCCAGCCCCCGATTGGGG-3′) or a primer for GAPDH (5′-TCTACATGGCAACTGTGA-3′), respect-ively. The transcription products of NS3 and GAPDH were amplified by PCR using paired primers (5′-ATGG CGCCTATTACGGCC-3′ and 5′-TGGTCTACATTAG TGTAC-3′) and (5′-GGTGGTCTCCTCTGACTTCAA CA-3′ and 5′-GTGGTCGTTGAGGGCAATG-3′), re-spectively. The putative sizes of the PCR products were 242-bp for NS3 and 89-bp for GAPDH. The PCR products were separated on 2% agarose gel.

RESULTS

RNA pol I-driven plasmid vector

First, we constructed an RNA pol I-driven plasmid coding an HCV replicon in which structural coding genes were replaced by the luciferase gene (Figure 1A). To investigate the expression of the HCV replicon from the RNA pol I plasmid vector, we transfected the plasmid vector into Huh7 cells. As shown in Figure 1B, the luciferase activity was observed in the RNA pol I vector-transfected cells. IFN is the most popular agent used to inhibit HCV replication. To examine whether the RNA pol I plasmid vector functions as an assay system for anti-HCV activity, we investigated the effect of IFN on the expression of the HCV replicon in the RNA pol I plasmid-transfected Huh7 cells. IFN dose-dependently reduced the replication of the HCV genome (Figure 1C), reaching 29.2% of the control at 5 pg/ml. IFN treatment did not cause any cytotoxicity (Figure 1D). These data suggest that the RNA pol I plasmid coding the HCV replicon works as an assay system for HCV replication.

RNA pol I-driven Ad5 vector

The Ad vector is the most efficient gene transfer vector for a variety of mammalian cells *in vitro* and *in vivo* (13,14,19,20). There are more than 51 serotypes of Ad. The Ad type 5 (Ad5) vector has been frequently used in basic research and clinical studies (21). Ad5 vectors are 100- and 1000-fold more efficient at mediating gene transduction than cationic lipids, an effective non-viral vector (22). A reverse genetics system for the generation of influenza virus using RNA pol I-driven Ad5 vector produced 1000-fold the virus titer of the RNA pol I plasmid system (15). These findings indicate that the Ad5 vector may have advantages for the preparation of an HCV replicon system. We prepared RNA pol I-driven Ad5 vectors and confirmed the expression of a reporter gene from the Ad5 vectors coding luciferase (Supplementary Figure S1). However, we did not succeed in preparing Ad5 vector particles coding the HCV replicon. Indeed, there have been no previous reports of the preparation of Ad5 vector expressing the HCV RNA genome.

We think that two problems must be solved in order to develop Ad5 vectors coding the HCV RNA genome. These problems are the influence of the HCV replicon on the preparation of Ad5 particles and the packaging limit of Ad5 vectors.

Preparation of the TRE/RNA pol I chimeric promoter

The tet-regulated system comprises a regulator vector that expresses tet-controlled *trans*-activators and a response vector consisting of TRE within the promoter that controls expression of the gene of interest. The tet-controlled *trans*-activators are classified into tTA and rtTA that binds to the TRE promoter and activates ex-pression from the TRE promoter in the absence and presence of Dox, respectively (23,24). We speculated that a tet-regulated vector system would minimize the influence of the HCV replicon on the preparation of Ad vector

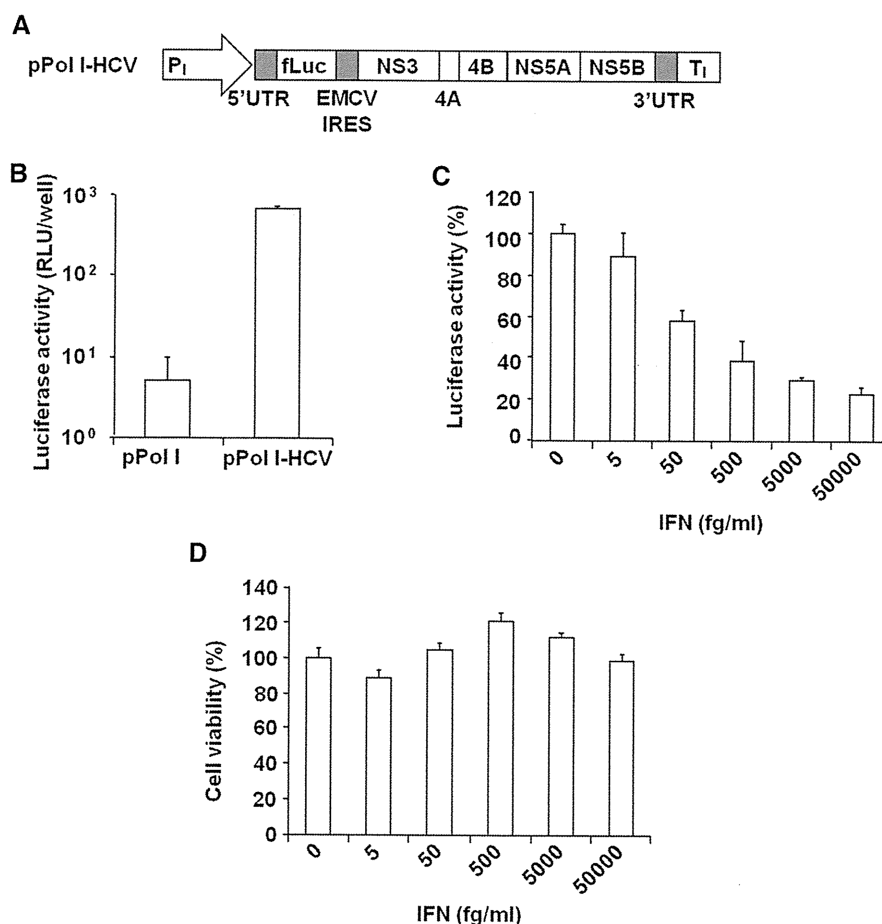

Figure 1. Preparation of plasmid expressing HCV replicon driven by RNA pol I promoter. (**A**) Schematic construct of HCV replicon-expression cassette. The HCV replicon gene was driven by the RNA pol I promoter (P_I) and terminator (T_I). (**B**) Transgene expression in Huh7 cells. Cells were transfected with pPol I-HCV. After 24 h of transfection, the luciferase activities were measured. Data are mean ± SD (n = 3). (**C** and **D**) Effect of IFN on HCV replication in RNA pol I vector-transfected cells. Huh7 cells were transfected with pPol I-HCV. After 2.5 h of transfection, the cells were treated with IFN at the indicated concentration. After an additional 72 h of incubation, the luciferase activity (C) and the cell viability (D) were measured. The luciferase activity (%) was calculated as a percentage of that in the vehicle-treated cells. Data are mean ± SD (n = 3).

particles. First, we optimized the chimeric promoter of TRE and the RNA pol I promoter. As shown in Figure 2A, the RNA pol I promoter is a 412-bp fragment containing an upstream control element (UCE) and the binding site of a transcription factor (Core). We constructed three chimeric promoter-driven plasmid vectors and checked the expression profiles using luciferase as a reporter gene. The chimeric vector was co-transfected into Huh7 cells with response vectors coding tTA or rtTA (23,24). As shown in Figure 2B and C, co-transfection with tTA exhibited a higher expression level than that of rtTA. The P_I235 promoter had the lowest luciferase expression in the absence of response vectors (Supplementary Figure S2). We used tTA and the P_I235 promoter in further studies. To investigate whether the chimeric RNA pol I promoter works in the Ad vector, we prepared Ad5 vector coding the chimeric RNA pol I-driven luciferase gene. AdP_I235-EL (MOI of 5) was co-transduced with Ad-tTA at MOI of 10 and 50. As shown in Figure 2D, the luciferase expression was increased in an Ad-tTA concentration-dependent manner.

Expression of the HCV replicon from Ad vector

The packaging limit of a foreign gene in the conventional Ad5 vector has been estimated to be 8.1–8.2-kb (25). The HCV replicon is ~8.9-kb and contains a 1.7-kb firefly luciferase gene and sequence derived from the HCV genome. Thus, another reason for no previous reports regarding the preparation of Ad5 vector coding the HCV replicon appears to be the packaging limit of the Ad5 vector. Mizuguchi and Hayakawa found that Ad5/35 vector containing chimeric fibers of Ad5 and Ad35 increased the size limit of foreign genes to 8.8-kb (26). We were successful in preparing Ad5/35 vector particles (9.53×10^8 IFU/ml) coding the TRE/RNA pol I chimeric promoter-driven HCV replicon containing the 1.0-kb renilla luciferase gene and sequence derived from the HCV genome (Figure 3A). To investigate the expression of the HCV replicon, Huh7 cells were transfected with the Ad vector coding the HCV replicon and Ad-tTA at MOI of 10 and 50, respectively. As shown in Figure 3B, western blot analysis showed that NS5A was expressed in Huh7 cells transfected with the vectors in the absence of Dox.