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A Novel Tumor-Targeted Therapy Using a Claudin-4-Targeting Molecule

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ABSTRACT

Carcinogenesis is often accompanied by dysfunctional tight junction (TJs), resulting in the loss of cellular polarity. Claudin, a tetra-transmembrane protein, plays a pivotal role in the barrier and fence functions of TJs. Claudin-4 is deregulated in various cancers, including breast, prostate, ovarian, and gastric cancer. Claudin-4 may be a promising target molecule for tumor therapy, but the claudin-targeting strategy has never been fully developed. In the present study, we prepared a claudin-4-targeting molecule by fusion of the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) with the protein synthesis inhibitory factor (PSIF) derived from *Pseudomonas aeruginosa* exotoxin. PSIF was not cytotoxic to claudin-4-expressing cells, whereas C-CPE-PSIF was cytotoxic. Cells that express claudin-1, -2, and -5 were less sensitive to C-CPE-PSIF. Pretreatment of the cells with C-CPE attenuated C-CPE-PSIF-induced cytotoxicity, and mutation of C-CPE in the claudin-4-binding residues attenuated the cytotoxicity of C-CPE-PSIF. TJ-undeveloped cells were more sensitive to C-CPE-PSIF than TJ-developed cells. It is noteworthy that polarized epithelial cells are sensitive to C-CPE-PSIF applied to the basal side, whereas the cells were less sensitive to C-CPE-PSIF applied to the apical side. Intratumoral injection of C-CPE-PSIF reduced tumor growth. This is the first report to indicate that a claudin-4-targeting strategy may be a promising method to overcome the malignant tumors.

The majority of lethal cancers are derived from epithelial tissues (Jemal et al., 2008), and various therapeutic strategies against epithelium-derived cancers have been developed. Targeted therapies that use differences between normal cells and cancer cells are promising antitumor therapies, and cellular surface proteins displayed on cancer cells are often targeted. Genetically modified toxins that target the surface proteins have emerged as a promising treatment strategy for refractory cancers (Michl and Gress, 2004). However, malignant tumors are still a major cause of death; more than 7 million people worldwide die from cancer each year (Dunham, 2007). Thus, the development of a novel strategy for cancer-targeting therapy is needed.

A defining feature of epithelial cells is cellular polarity. Epithelial cells have tight junctions (TJs) on the membrane between adjacent cells. TJs seal the intercellular space between adjacent cells and regulate solute movement across epithelial cell sheets (Anderson and Van Itallie, 1995). In addition, TJs form the fence of the membrane that prevents lateral diffusion of membrane proteins and lipids, thereby maintaining the differential composition of the apical and basolateral domains. TJs also seem to be involved in the regulation of proliferation, differentiation, and other cellular

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ABBREVIATIONS: TJ, tight junction; C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin from 194 to 319 amino acids; PSIF, protein synthesis inhibitory factor derived from *P. aeruginosa* exotoxin; CPE, *Clostridium perfringens* enterotoxin; PE, *P. aeruginosa* exotoxin; C-CPE-PSIF, C-terminal fragment of *Clostridium perfringens* enterotoxin-fused protein synthesis inhibitory factor derived from *P. aeruginosas* exotoxin; PAGE, polyacrylamide gel electrophoresis; BV, budded baculovirus; Ab, antibody; TER, transepithelial electric resistance; FBS, fetal bovine serum; MCS, multiple cloning sites; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TBS, Tris-buffered saline; LDH, lactate dehydrogenase; aa, amino acid.

functions (Mitic and Anderson, 1998; Vermeer et al., 2003). Malignant tumor cells frequently exhibit abnormal TJ function, followed by the loss of cellular polarity and intercellular contact that commonly occurs in advanced tumors and early stages of carcinogenesis (Wodarz and Näthke, 2007). These findings indicate that TJ proteins, which are barely accessible in well structured normal epithelia but are exposed on malignant tumor cells, may be promising candidates for targeted therapy.

Freeze-fracture replica electron microscopy analysis reveals that TJs appear as a series of continuous, anastomotic, and intramembranous particle strands or fibrils (Tsukita and Furuse, 1999). The TJ strands consist of integral membrane proteins polymerizing linearly within a lipid layer of the cell membrane. Claudin, a ~24-kDa four-transmembrane protein that forms a family containing 24 members, is a crucial component of the TJ strand in forming the TJ fence (Furuse and Tsukita, 2006). The expression of claudins is altered in several cancers. In particular, claudin-4 is frequently upregulated in breast, prostate, pancreatic, and ovarian cancers (Morin, 2005). Thus, the claudins are potential targets for antitumor therapy. Although antibodies to the extracellular region of claudins are promising molecules for tumor-targeted therapy, these antibodies have not yet been successfully prepared.

Clostridium perfringens enterotoxin (CPE) is a single polypeptide of 35-kDa that can cause food poisoning in humans. Functional domains of CPE are classified into the N-terminal cytotoxic region and the C-terminal receptor-binding region (Hanna et al., 1992). CPE binds to its receptor, claudin-4, followed by the formation of the complex on the membrane leading to cell damage (Katahira et al., 1997; Paperna et al., 1998; McClane and Chakrabarti, 2004). Prostate adenocarcinoma cells expressing claudin-4 are sensitive to CPE-mediated cytolysis (Long et al., 2001). Breast, ovarian, and pancreatic cancer cells expressing claudin-4 are also sensitive to CPE treatment (Michl et al., 2001; Kominsky et al., 2004; Santin et al., 2005). These findings suggest that the receptor-binding region of CPE is useful for targeting claudin-4-expressing cancer cells.

Pseudomonas aeruginosa exotoxin A (PE) is widely used in cancer-targeting therapy. PE binds to the cell surface and is internalized via an endocytotic pathway, followed by escape of the PE fragment (protein synthesis inhibitory factor, PSIF) from the endosome into the cytosol. The released PSIF inhibits protein synthesis by the inhibition of elongation factor 2 (Ogata et al., 1990). PSIF lacks the receptor binding domain of PE, and fusion of the ligand of tumor antigen with PSIF is a promising strategy for cancer-targeting therapy. For example, the fusion protein of PSIF with anti-interleukin-2 antibody has been used in clinical therapy (Kreitman and Pastan, 2006). In the present study, we genetically prepared the claudin-4-targeting molecule (C-CPE-PSIF) containing the claudin-4-binding region of CPE and PSIF, and we found that C-CPE-PSIF has in vivo antitumor activity.

Materials and Methods

Cell Culture. Mouse fibroblast cell line L cells and mouse claudin-expressing L cells (claudin-1/L, -2/L, -4/L, and -5/L cells), kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan), were cultured in modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Human hepatocarcinoma cell lines HepG2 cell and SK-HEP-1 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS. The human intestinal cell line Caco-2 was maintained in Dulbecco's modified Eagle's medium containing 10% FBS and 1% nonessential amino acids. The murine mammary carcinoma cell line 4T1 was maintained in Dulbecco's modified Eagle's medium containing 10% FBS and 10 mM HEPES. The cells were maintained in a 5% CO_2 atmosphere at 37°C.

Preparation of C-CPE-PSIF. We prepared plasmids containing the C-terminal CPE-fused PSIF. In brief, we generated pET16b-MCS. Double-stranded oligonucleotides of MCS were prepared by annealing (heating at 95°C for 5 min and chilling at room temperature for 60 min) single-strand oligonucleotides, a forward oligonucleotide (5′-TCGAAGGTACCCGGGACTAGTTAATTAAG-3′, XhoI binding site is underlined) and a reverse oligonucleotide (5′-TCGAACTTTTATAACTAGTCCCGGGTCCAT-3′, XhoI binding site is underlined). The annealed oligonucleotides were subcloned into the pET16b vector (Novagen, Darmstadt, Germany), resulting in pET16b-MCS. PSIF was amplified by polymerase chain reaction (PCR) using pPBV-PE40 as a template, a forward primer (5′-GATGATCTGAGCGGCCGCAACCCGAGGGCGGCAG-3′, NotI site is underlined), and a reverse primer (5′-TCCAGATCTTTACAGTTCGTCTTTCTTCAGGTCCTC-3′, BglII site is underlined). The resulting PCR fragments were subcloned into NotI/BamHI-digested pET16b-MCS to create pET-PSIF, and the sequence was confirmed. C-CPE and C-$CPE_{Y306A/L315A}$ were amplified by PCR with $pETH_{10}PER$ as a template. The common forward primer is (5′-GGAATTCCATATGGATATAGAAAAAGAAATCCTTGATTTAGCTGCT-3′, SpeI site is underlined), and the reverse primer for C-CPE is (5′-GGACTAGTAAATTTTTGAAATAATATTGAATAAGGGTAATTTCCACTATATG-3′, NdeI site is underlined) or C-$CPE_{Y306A/L315A}$ (5′-GGACTAGTAAATTTTTTGCTATTGAATAAGGGTAATTTCCACTAGCTGATGAATTAGCTTTCATTAC-3′, NdeI site is underlined). The resulting PCR products were subcloned into SpeI/NdeI-digested pET-PSIF to create pET-C-CPEs-PSIF, and the sequence was confirmed. The plasmids, pET-PSIF and pET-C-CPEs-PSIF, were transduced into *Escherichia coli* BL21 (DE3) strains (Novagen), and the production of PSIF and C-CPE-PSIF was induced by the addition of 0.25 mM isopropyl-D-thiogalactopyranoside. The cells were harvested and then lysed in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol). The lysates were centrifuged, and the resultant supernatant was applied to HiTrap Chelating HP (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The proteins were eluted by imidazole in buffer A. The buffer was exchanged with phosphate-buffered saline (PBS) by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80°C until use. Protein was quantified by using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin (BSA) as a standard.

Cytotoxic Activity. In the cytotoxic assay, L-cells were seeded onto a 96-well culture dish at 10^4 cells/well. After 24 h of the culture, the cells were treated with PSIF or C-CPE-PSIF for 24 h at the indicated concentration. HepG2, SK-HEP-1, and 4T1 cells were seeded onto a 96-well culture dish at 10^4 cells/well. After 24 h of culture, the cells were treated with PSIF or C-CPE-PSIF for 48 h at the indicated concentration. In a preconfluent assay, Caco-2 cells were seeded onto a 96-well culture dish at 10^4 cells/well. After 24 h of culture, the cells were treated with PSIF or C-CPE-PSIF for 48 h at the indicated concentration. In a postconfluent assay, Caco-2 cells were cultured in a 96-well culture dish for an additional 3 days after reaching a confluent condition. Then, the cells were treated with PSIF or C-CPE-PSIF for 48 h at the indicated concentration. The cytotoxicity was determined by a WST-8 kit according to the manufacturer's instructions (Nacalai Tesque, Kyoto, Japan).

Immunoblot Analysis. Cells were lysed in lysis buffer [20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and phosphatase inhibitor cocktail (Nacalai)]. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The separated proteins were

transferred onto a polyvinylidene difluoride membrane, followed by immunoblotting with anti-claudin-1, -2, -4, and -5 (Zymed Laboratories, South San Francisco, CA), anti-His-tag (Novagen), or anti-β-actin Ab (Sigma-Aldrich). The immunoreactive band was visualized by chemiluminescence reagents (GE Healthcare).

Competition Assay. After pretreatment of claudin-4/L (CL4/L) cells with C-CPE or BSA for 2 h, the cells were incubated with C-CPE-PSIF for 24 h. Then the cell viability was assayed by the cell counting kit, as described above.

Preparation of the Claudin-Displaying Budded Baculovirus. The cDNAs for mouse claudin-1 and claudin-4 were amplified by PCR from pGTCL-1 and pGTCL-4 (kindly provided by Dr. M. Furuse, Kobe University, Kobe, Japan). The DNA fragments were subcloned into the baculoviral transfer vector pFastBac1 (Invitrogen, Carlsbad, CA). Recombinant baculoviruses were generated by using the Bac-to-Bac system (Invitrogen) according to the manufacturer's instructions.

Sf9 cells were cultured in Grace's Insect medium (Invitrogen) containing 10% FBS at 27°C. Sf9 cells were infected with the recombinant baculovirus. Seventy-two hours after infection, the budded baculovirus (BV) fraction was isolated from the culture supernatant of infected Sf9 cells by centrifugation at 40,000*g* for 25 min. The pellets of the BV fraction were suspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail and then stored at 4°C. The expression of claudins in the BV fraction was confirmed by SDS-PAGE and immunoblot analysis.

Enzyme-Linked Immunosorbent Assay. The BV-displaying claudins were diluted with TBS and adsorbed to the wells of 96-well immunoplates (Nunc, Roskilde, Denmark) overnight at 4°C. The wells were washed with PBS and blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) for 2 h at room temperature. C-CPEs-PSIF was added to the well and incubated for an additional 2 h at room temperature. The wells were washed with PBS and incubated with anti-His-tag antibody for 2 h at room temperature. The immunoreactive proteins were detected by horseradish peroxidase-labeled secondary antibody by using 3,3',5,5'-tetramethyl benzine as a substrate. The reaction was terminated by the addition of 0.2 M H_2SO_4, and the immunoreactive proteins were measured at 450 nm.

Measurement of Transepithelial Electric Resistance. Confluent monolayers of Caco-2 cells were grown in Transwell chambers (Corning Life Sciences, Lowell, MA). The formation of tight junction barriers in Caco-2 monolayers was monitored by measuring transepithelial electric resistance (TER) with a Millicell-ERS epithelial volt-ohmmeter (Millipore Corporation, Billerica, MA). After 7 to 10 days of culture, the TER values reached a plateau. Then, C-CPE-PSIF was added to the apical or basolateral compartment of the Transwell chamber, and the TER values were measured for 48 h. The TER values were normalized by the area of the Caco-2 monolayer. The background TER of a blank Transwell chamber was subtracted from the TER of cell monolayers.

L-Lactate Dehydrogenase Release Assay. The release of lactate dehydrogenase (LDH) from cells was analyzed by using a Cyto-Tox-96 NonRadioactive Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. LDH release was calculated by using the following equation: % maximal LDH release = LDH in the cultured medium/total LDH in the culture dish.

Reverse Transcriptase-Polymerase Chain Reaction. Total RNA was isolated with a High Pure RNA Isolation Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions, and the RNA was treated with RNase-free DNase. Then, 200 ng of RNA was reverse-transcribed with dT adaptor by using a Takara RNA PCR Kit (Takara Inc., Shiga, Japan), according to the manufacturer's instructions. The resulting cDNA was amplified by Ex Taq DNA polymerase (Takara). The PCR conditions were as follows: 96°C for 2 min, 30 cycles of 96°C for 45 s, 55°C for 60 s, and 72°C for 30 s. Claudin-4 and β-actin mRNA were detected by using the following primers: forward primer, 5'-ATGGCGTCTATGGGACTACAGGTCC-3'; reverse primer, 5'-CCGAGTAGGGCTTGTCGTTGCTAC-3'; and forward primer, 5'-TAGATGGGCACAGTGTGGG-3'; reverse primer, 5'-GGCGTGATGGTGGGCATGG-3', respectively.

In Vivo Antitumor Activity. Female BALB/c mice (7–8 weeks old) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). The mice were housed in an environmentally controlled room at 23 ± 1.5°C with a 12-h light/dark cycle. 4T1 cells (2×10^6) in 50 μl of PBS were injected subcutaneously into the right flank on day 0. Tumor size was determined by measuring two diameters, and the tumor volume was calculated by the following equation: tumor volume = $a \times b \times b/2$, where a represents the maximum tumor diameter and b represents the minimum tumor diameter. After the inoculation of the cells, intratumoral injection of PBS, C-CPE, C-CPE$_{Y306A/L315A}$-PSIF, or C-CPE-PSIF was performed on days 2, 4, 7, 9, 11, and 14.

Statistical Analysis. Data were analyzed by using two-way analysis of variance followed by the Student's t test. The statistical significance for all comparisons was set at $p < 0.05$.

Results

Preparation of C-CPE-PSIF. Overexpression of claudin-4 is frequently observed in malignant tumors, indicating that the claudins are promising proteins for cancer-targeting therapy (Morin, 2005). We previously developed a novel drug delivery system by using a claudin-4-targeting molecule, the C-terminal of CPE corresponding to 184 to 319 aa (C-CPE$_{184-319}$) (Kondoh et al., 2005; Ebihara et al., 2006). Because of its poor solubility (less than 0.3 mg/ml), it is difficult to use C-CPE$_{184-319}$ in pharmaceutical therapy. Van Itallie et al. (2008) reported that the deletion of the N-terminal 10 aa of C-CPE$_{184-319}$, resulting in C-CPE$_{194-319}$, maintains the claudin binding activity of C-CPE$_{184-319}$ and has high solubility (more than 10 mg/ml) (Van Itallie et al., 2008). We prepared claudin-targeting antitumor agents by genetic fusion of C-CPE$_{194-319}$ and PSIF (C-CPE-PSIF) as shown in Fig. 1A. Here, C-CPE refers to C-CPE$_{194-319}$. PSIF and C-CPE-PSIF were produced by *E. coli* and were purified by affinity chromatography by using a histidine tag. Purification of the proteins was confirmed by SDS-PAGE or immunoblotting (Fig. 1B). The molecular size as determined by SDS-PAGE was identical with the predicted size (C-CPE-PSIF, 60 kDa).

Characterization of C-CPE-PSIF. To evaluate the binding of C-CPE-PSIF to claudins, we used a BV display system. In this system, exogenous proteins are expressed on the surface of the virus particle retaining its function (Sakihama et al., 2008). CPE did not bind to claudin-1 (Fujita et al., 2000). We generated claudin-1-BV and claudin-4-BV and confirmed the claudin expression on BV by Western blotting (data not shown). After the claudin-BV was adsorbed on wells, C-CPE, C-CPE-PSIF, and PSIF proteins were added. We detected the binding of proteins to BV by using anti-histidine-tag antibody. As shown in Fig. 2A, C-CPE specifically interacted with claudin-4-BV but not with wild-type or claudin-1-BV. C-CPE with a mutation on the claudin-4-binding region did not interact with claudin-4-BV (data not shown). These data suggest that fusion with C-CPE provides claudin-4-binding activity to PSIF.

To assess whether C-CPE-PSIF is cytotoxic to claudin-4-expressing cells, we treated claudin-4/L cells with PSIF or C-CPE-PSIF for 24 h. C-CPE-PSIF dose-dependently caused cell death, reaching >90% cell death at 10 ng/ml (Fig. 2B). In contrast, PSIF was not cytotoxic, even at 20 ng/ml. We performed a competition assay to determine whether C-CPE-

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PSIF binds to claudin-4 via C-CPE. When the cells were pretreated with BSA or C-CPE before C-CPE-PSIF treatment, C-CPE dose-dependently attenuated the cytotoxic activity of C-CPE-PSIF (Fig. 2C). BSA did not affect the activity even by pretreatment at 10 μg/ml, at which C-CPE completely attenuated the cytotoxicity of C-CPE-PSIF. These results suggest that C-CPE-PSIF interacts with claudin-4 via the C-CPE domain.

The claudin family contains 24 members (Furuse and Tsukita, 2006). CPE containing C-CPE as a receptor binding domain bound to claudin-3, -4, -6, and -9 but did not bind to claudin-1, -2, or -5 (Fujita et al., 2000). To confirm the claudin-specificity of C-CPE-PSIF, we investigated the cytotoxic activity of C-CPE-PSIF in L cells expressing claudin-1, -2, -4, or -5 (Fig. 2D). As shown in Fig. 2E, claudin-1/L, -2/L and -5/L cells were less sensitive to C-CPE-PSIF than claudin-4/L cells. Taken together, these results indicate that C-CPE-PSIF may be a claudin-4-targeting cytotoxic agent.

Specificity of C-CPE-PSIF on Cytotoxicity. As mentioned above, C-CPE-PSIF is toxic to exogenous claudin-4-expressing L cells. To examine the specificity of C-CPE-PSIF in the cell lines, we checked the expression of claudin-4 in some human cell lines by immunoblotting and selected two human hepatocarcinoma cell lines: claudin-4-positive HepG2 cells and claudin-4-negative SK-HEP-1 cells (Fig. 3A). Human breast cancer cell line MCF-7 cells were used as a positive control (Ebihara et al., 2006). C-CPE-PSIF was toxic to HepG2 cells and MCF-7 cells, reaching 89 and 53% cell death at 1 and 100 ng/ml, respectively. In contrast, C-CPE-PSIF was not toxic to SK-HEP-1 cells, even at 100 ng/ml (Fig. 3B). Thus, C-CPE-PSIF may have specific toxicity to claudin-4-expressing cells.

Claudin-4 is expressed in various tissues, such as lung, intestine, liver, and kidney. Most claudins in normal cells would be contained in TJ complexes, whereas the localization of claudins is deregulated in some cancers (Morin, 2005; Kominsky, 2006). C-CPE-PSIF may recognize the deregulated localization of claudin-4, resulting in less toxicity to normal cells. We next examined the effects of C-CPE-PSIF on the human colon carcinoma cell line Caco-2, which expresses claudin-4. Caco-2 cells form a polarized cell monolayer with well developed TJs when they reach confluence, and they are frequently used as a model of polarized cells (Meunier et al., 1995). The claudin-4 protein levels in the confluent culture were greater than the levels in the subconfluent culture (Fig. 3C). As shown in Fig. 3D, C-CPE-PSIF was toxic in the preconfluent cells with fewer TJs (47% cell death at 5 ng/ml). In contrast, the postconfluent cells with well developed TJs were less sensitive to C-CPE-PSIF than the preconfluent cells, and treatment of the cells with C-CPE-PSIF resulted in 40% cell death, even at 200 ng/ml (Fig. 3D).

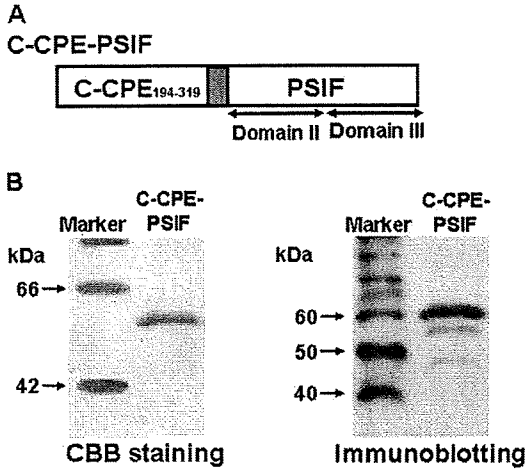

Fig. 1. Preparation of C-CPE-PSIF. A, schematic structure of C-CPE-PSIF. C-CPE-PSIF is a fusion protein of C-CPE and PSIF. C-CPE is the C-terminal fragment of CPE 194 to 319 aa (Van Itallie et al., 2008). The dark area indicates the putative receptor-binding region of C-CPE located in its C terminus (Takahashi et al., 2005). PSIF contains domain II and III of PE. Domain II is critical for the escape of the toxin from the endosome to the cytosol, and domain III is responsible for the inhibition of protein synthesis (Ogata et al., 1990). B, purification of PSIF and C-CPE-PSIF. C-CPE-PSIF was expressed in *E. coli* and isolated by nickel-affinity chromatography. The purification of proteins (5 μg) was confirmed by SDS-PAGE followed by immunoblotting with antibody against the histidine tag. The putative molecular mass of C-CPE-PSIF is approximately 60 kDa.

Early events in epithelial carcinogenesis are the deregulation of cellular polarity and the loss of TJ structures (Wodarz and Näthke, 2007). Next, we examined whether C-CPE-PSIF sensitivity is affected by the cellular polarity in Caco-2 monolayer cell sheets grown on the membrane in Transwell chambers. The Caco-2 monolayer cells exhibit a well differentiated brush border containing TJs on the apical surface, and they are frequently used as an epithelial cell sheet model (Meunier et al., 1995). After the addition of C-CPE-PSIF in the apical or basolateral compartment of the Transwell chamber, we checked the TJ barrier function of the cell sheets by measuring the TER. When C-CPE-PSIF was added to the apical compartment, the TER was not affected for 48 h. In contrast, the addition of C-CPE-PSIF to the basolateral compartment caused a significant reduction in the TER in a dose-dependent manner (Fig. 3E). Furthermore, we found that the addition of C-CPE-PSIF to the basolateral compartment, but not the apical compartment, increased the amount of released LDH, a marker of cytotoxicity (Fig. 3F). Thus, C-CPE-PSIF may have specificity to the cellular polarity.

In Vivo Antitumor Activity of C-CPE-PSIF. We preliminarily investigated the expression of claudin-4 and tumor formation in conventional mice, and we selected a mouse breast cancer cell line, 4T1. Reverse transcription-polymerase chain reaction and immunoblotting analysis revealed the expression of claudin-4 in 4T1 cells (Fig. 4A). To clarify the antitumor activity of a claudin-4-targeting molecule, C-CPE-PSIF, we prepared a fusion protein of PSIF with mutant C-CPE, in which two residues critical for the interaction between C-CPE and claudin-4 (Tyr306 and Leu315) were changed to alanines (Takahashi et al., 2008), resulting in C-CPE$_{Y306A/L315A}$-PSIF (Fig. 4B). C-CPE$_{Y306A/L315A}$-PSIF lost the claudin-4-binding activity and was not toxic to CL4/L cells (Fig. 4, C and D). C-CPE-PSIF had dose-dependent cytotoxicity in 4T1 cells, reaching 63% cell death at 100 ng/ml. In contrast, C-CPE$_{Y306A/L315A}$-PSIF did not show any cytotoxicity, even at 500 ng/ml, indicating that the cytotoxicity of C-CPE-PSIF in 4T1 cells may be mediated by its binding to claudin-4 (Fig. 4E).

Next, to investigate the in vivo antitumor activity of C-CPE-PSIF, 4T1 cells (2×10^6 cells) were inoculated into the right flank of mice on day 0. On day 2, the volume of the tumor exceeded 17 mm^3. Vehicle, C-CPE, C-CPE-PSIF, or C-CPE$_{Y306A/L315A}$-PSIF was intratumorally injected on days

2, 4, 7, 9, 11, and 14 at 5 μg/kg. Injection of C-CPE-PSIF significantly suppressed tumor growth, resulting in 36% of the tumor volume in the vehicle-treated group on day 16 (Fig. 4F). In contrast, C-CPE and C-CPE$_{Y306A/L315A}$-PSIF lacking claudin-4-binding activity had no effect on tumor growth, indicating that the antitumor activity of C-CPE-PSIF may be dependent on the claudin-4 targeting. The body weight of the mice did not change after injection of C-CPE-PSIF (data not shown). The appearance of the C-CPE-PSIF-injected mice also indicated no side effects.

Discussion

In the present study, we prepared a claudin-4-targeting ligand C-CPE coupled to PSIF of PE. We found that C-CPE-PSIF was selectively toxic to claudin-4-expressing cells and showed in vivo antitumor activity against mouse breast cancer cells, indicating that C-CPE may be a useful tool for claudin-targeted therapy.

Side effects of antitumor agents are a pivotal issue in clinical therapy. To reduce side effects, therapies that specif-

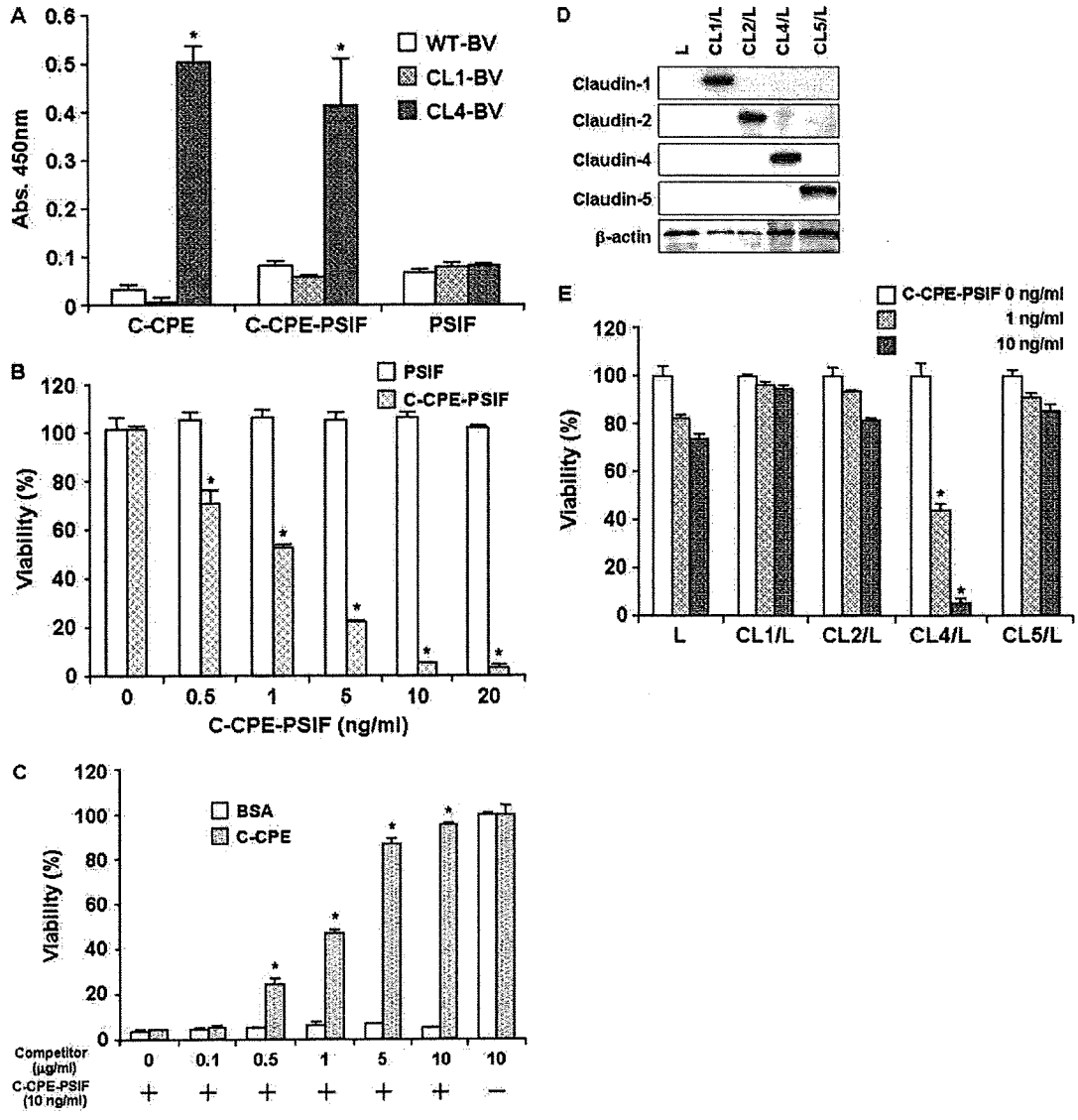

Fig. 2. Characterization of C-CPE-PSIF. A, interaction of C-CPE-PSIF with claudins. Wild-type BV (WT-BV), claudin-1-BV (CL1-BV), or claudin-4-BV (CL4-BV) was adsorbed onto a 96-well immunoplate at 0.5 μg/well. Then, 7.5 μmol C-CPE, C-CPE-PSIF, or PSIF was added to the well, and the protein bound to the BVs was measured by incubation of the anti-histidine-tag Ab, followed by horseradish peroxidase-labeled secondary Ab. The data represent the mean ± S.D. of three independent experiments. *, significantly different from the WT-BV value ($p < 0.01$). B, cytotoxicity of C-CPE-PSIF in claudin-4/L (CL4/L) cells. After a 24-h treatment of CL4/L cells with PSIF or C-CPE-PSIF at the indicated concentration, the cell viability was measured by WST-8 assay. Viability (percentage) was calculated as a percentage of the vehicle-treated cells. The data represent the mean ± S.D. of three independent experiments. *, significantly different from the vehicle-treated group ($p < 0.01$). C, competition assay using C-CPE. CL4/L cells were treated with C-CPE or BSA at the indicated concentration for 2 h, and then the cells were treated with C-CPE-PSIF (10 ng/ml) for 24 h. The cell viability was measured by WST-8 assay, as described above. The data represent the mean ± S.D. of three independent experiments. *, significantly different between BSA and C-CPE-treated groups ($p < 0.01$). D, immunoblot analysis. Lysates of L, CL1/L, CL2/L, CL4/L, or CL5/L cells were subjected to SDS-PAGE, followed by immunoblotting with antibodies against the indicated CL. E, specific cytotoxicity of C-CPE-PSIF. L, CL1/L, CL2/L, CL4/L, and CL5/L cells were treated with C-CPE-PSIF for 24 h at the indicated concentration. The cell viability was assayed by WST-8 assay. The data represent the mean ± S.D. of three independent experiments. *, significantly different from the L-cells ($p < 0.01$).

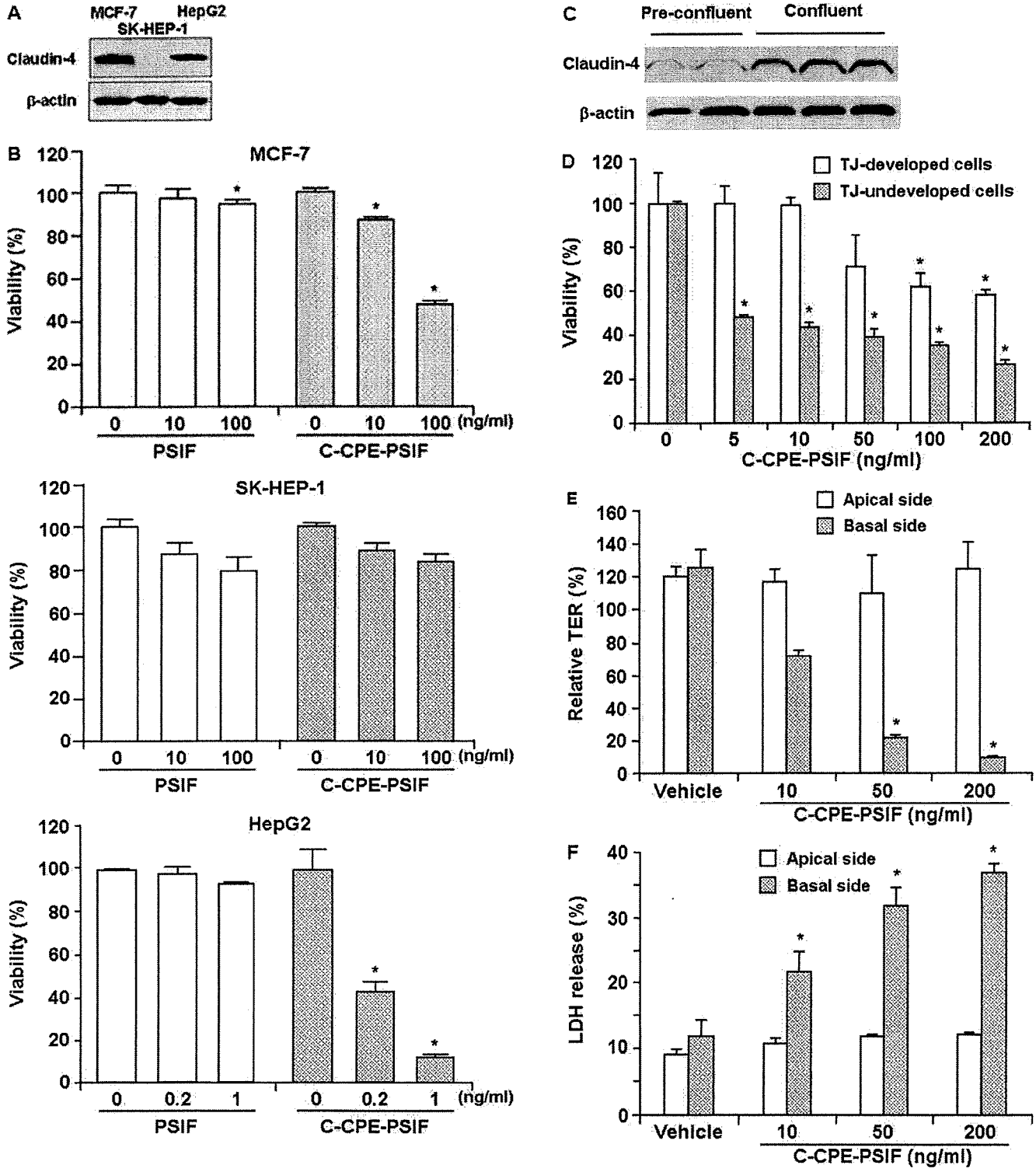

Fig. 3. Cytotoxic specificity of C-CPE-PSIF. A, expression of claudin-4 in SK-HEP-1 and HepG2 cells. The cell lysates were subjected to SDS-PAGE, followed by Western blotting with anti-claudin-4 Ab. MCF-7 cells were used as a positive control. B, cytotoxicity of C-CPE-PSIF in SK-HEP-1 and HepG2 cells. Cells were treated with PSIF or C-CPE-PSIF for 48 h at the indicated concentration. The cell viability was measured by WST-8 assay. The data are representative of at least three independent experiments. Data are the mean ± S.D. (n = 3). *, significantly different from the vehicle-treated cells ($p < 0.01$). C and D, cytotoxicity of C-CPE-PSIF in TJ-developed or -undeveloped Caco-2 monolayer cells. TJ-developed cells were Caco-2 monolayer cells grown at confluence for 3 days. TJ-undeveloped cells were Caco-2 cells seeded at 10^4 cells/well in 96-well plates. The cell lysates were subjected to SDS-PAGE, followed by Western blotting with anti-claudin-4 Ab (C). The cells were treated with the indicated concentrations of C-CPE-PSIF for 48 h, and then the cell viability was measured as above (D). The data are representative of at least three independent experiments. Data are the mean ± S.D. (n = 3). *, significantly different from the vehicle-treated cells ($p < 0.05$). E and F, effect of C-CPE-PSIF on TER and LDH release in Caco-2 monolayer cells. Caco-2 cells were grown on Transwell chambers to form tight junctions. When TER values were constant, C-CPE-PSIF was added to the apical or basolateral side in Transwell chambers at the indicated concentrations. After 0 and 48 h of incubation, TER values were measured (E), and the LDH release from the cell was determined (F). TER values and LDH release were calculated as the ratio of TER values at 0 h and of the total cellular LDH, respectively. The data are representative of at least three independent experiments. Data are the mean ± S.D. (n = 3). *, significantly different from the vehicle-treated cells ($p < 0.05$).

ically target tumors are needed. Rapid advances in molecular biology and proteomics have allowed the identification of tumor-specific targets for cancer therapy. Therapy that exploits tumor-specific targets has the advantages of high specificity and low systemic toxicity relative to standard chemotherapy (Waldmann, 1991; Allen and Cullis, 2004). Target molecule selection is critical to the success of targeted therapy. Most targeted therapies have been directed against growth factor receptors, such as ErbB2, which are overexpressed in cancers and are easily accessible because of their cell-surface localization (Zumkeller and Schofield, 1995; Deckert, 2009). A claudin-targeting strategy may have at

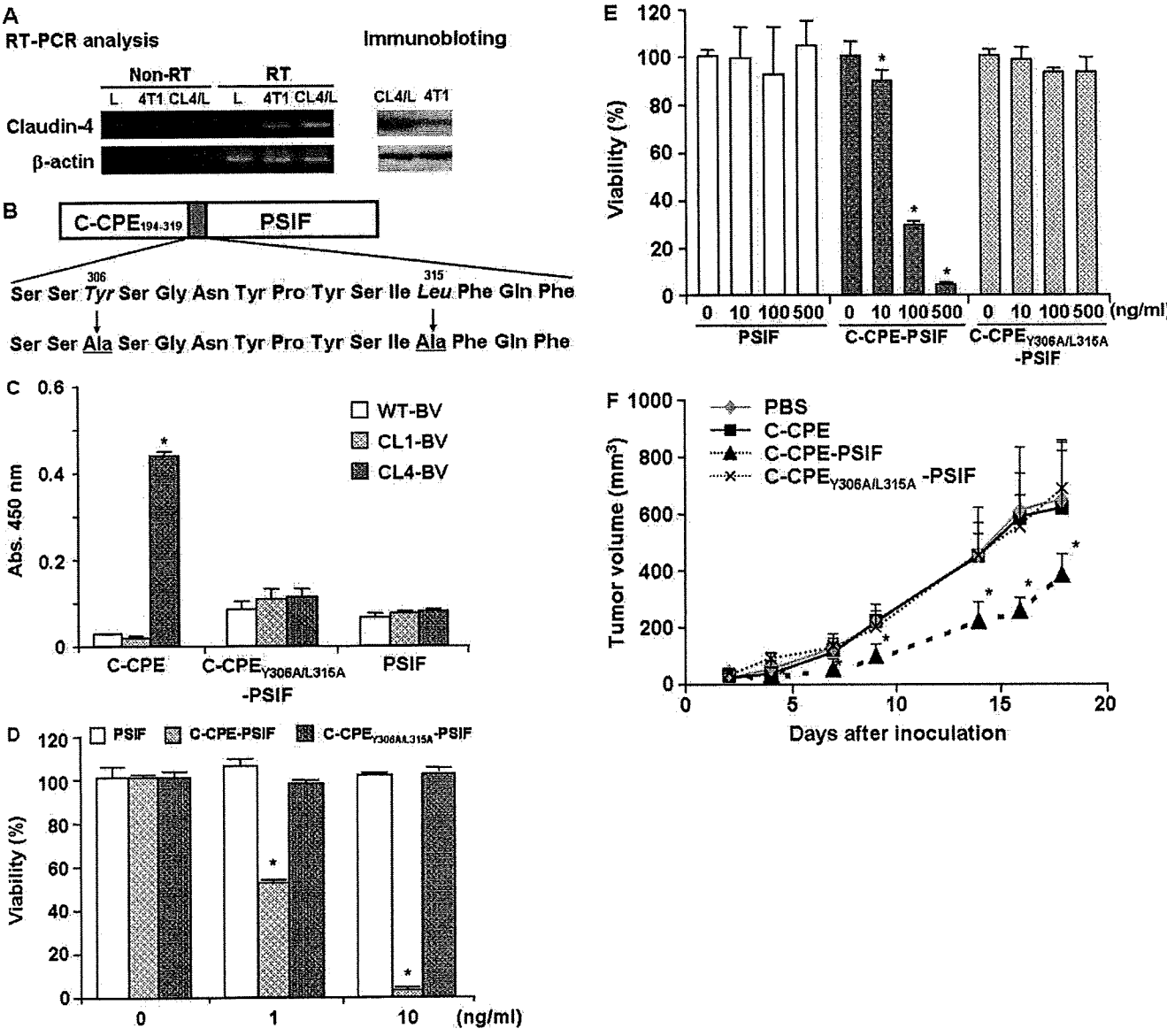

Fig. 4. In vivo antitumor activity of C-CPE-PSIF. A, expression of claudin-4 in 4T1 cells. Total RNA was extracted from the cells, followed by reverse transcription-polymerase chain reaction analysis (left). Cell lysates were subjected to SDS-PAGE, followed by Western blotting (right). β-Actin is an endogenous control. B, schematic structure of C-CPE$_{Y306A/L315A}$-PSIF. The dark area indicates the putative receptor-binding region of C-CPE in its C terminus (Takahashi et al., 2005). Among 16 amino acids, Tyr306 and Leu315 (indicated by italic letters) play a pivotal role in the binding of C-CPE with claudin-4 (Takahashi et al., 2008). C, interaction of C-CPE$_{Y306A/L315A}$-PSIF with claudin-4. WT-BV, CL1-BV, or CL4-BV was immobilized onto a 96-well immunoplate at 0.5 μg/well. Then, 7.5 μmol of C-CPE, C-CPE$_{Y306A/L315A}$-PSIF, or PSIF was added to the well, and the binding of the proteins to BVs was measured as described in the legend to Fig. 2A. The data represent the mean ± S.D. of three independent experiments. *, significantly different from the WT-BV value ($p < 0.01$). D, cytotoxicity of C-CPE$_{Y306A/L315A}$-PSIF in CL4/L cells. After a 24-h treatment of CL4/L cells with PSIF, C-CPE-PSIF, or C-CPE$_{Y306A/L315A}$-PSIF at the indicated concentration, the cell viability was measured as described in the legend to Fig. 2B. The data represent the mean ± S.D. of three independent experiments. *, significantly different from the vehicle-treated group ($p < 0.01$). E, cytotoxicity of C-CPE-PSIFs in 4T1 cells. Cells were treated with PSIF, C-CPE-PSIF, or C-CPE$_{Y306A/L315A}$-PSIF for 48 h at the indicated concentration. The cell viability was measured as above. The data represent the mean ± S.D. of three independent experiments. *, significantly different from the vehicle-treated group ($p < 0.05$). F, in vivo antitumor activity of C-CPE-PSIF. 4T1 cells (2×10^6 cells) were intradermally inoculated into the right flank of mice on day 0, and each sample (5 μg/kg) was intratumorally injected on days 2, 4, 7, 9, 11, and 14. Tumor growth was monitored by calculating tumor volume. The data are representative of three independent experiments. Each point is the mean ± S.D. from five mice. *, significantly different from the vehicle (PBS)-treated group ($p < 0.05$).

least two advantages over the previous targeted methods. First, the claudin-targeting method can recognize different cellular localizations of claudin between normal epithelium and malignant tumors. The expression of claudin is altered in several cancers (Nichols et al., 2004; Morin, 2005; Tsukita et al., 2008). It is noteworthy that claudin-4 is localized to the basolateral cell membrane at sites of cell-cell contact in normal epithelium; in contrast, claudin-4 is localized to the cell membrane even at sites that lack cell-cell contact in poorly differentiated human breast cancers (Kominsky, 2006). The claudin-targeting strategy might have low side effects. Indeed, claudin-targeting therapy using CPE had antitumor activities without side effects in cancers of the breast, ovary, prostate, and pancreas (Long et al., 2001; Michl et al., 2001; Rangel et al., 2003; Kominsky et al., 2004; Santin et al., 2005). Second, a claudin-targeting molecule may be a promising agent for solid tumors. Interstitial pressure is higher in the center of a tumor, and it approaches normal physiological pressure toward the periphery (Jain, 1987, 1989). High-pressure regions usually coincide with regions of poor perfusion and lower vessel surface area; as a result, it is difficult for antitumor agents to be delivered to the intratumor tissue. If an antitumor agent has penetration-enhancing activity, the agent will be effective in therapy for solid tumors. We previously found that C-CPE enhanced intestinal permeability of a drug by modulation of the claudin-claudin interaction. Binding of C-CPE with claudin-4 is critical for modulation of the interaction. Together, C-CPE-PSIF may enhance the intratumoral permeation of antitumor agents by modulation of the claudin-claudin interaction. Combination therapy of a chemical agent with a claudin-4-targeting agent will have a synergistic effect compared with that of a chemical agent alone.

In a previous study, we prepared C-CPE$_{184-319}$-PSIF, in which C-CPE corresponding to 184 to 319 amino acids of CPE was used, and we found that C-CPE$_{184-319}$-PSIF is potently cytotoxic to claudin-4-expressing cells (Ebihara et al., 2006). However, C-CPE$_{184-319}$-PSIF had poor solubility (0.3 mg/ml) and low cytotoxicity (IC_{50} = 2–3 μg/ml in claudin-4/L cells) (Ebihara et al., 2006). In 2008, Van Itallie et al. (2008) found that C-CPE$_{194-319}$ (referred to as C-CPE in the present study), which corresponds to amino acids 194 to 319 of CPE, is a claudin-4 binder with high solubility (10 mg/ml). We prepared a claudin-4-targeting molecule using C-CPE and found that C-CPE-PSIF has high solubility (~1.0 mg/ml) and high cytotoxicity (IC_{50} = 1 ng/ml in claudin-4/L cells). These results indicate that C-CPE is a promising claudin-4-targeting molecule for pharmaceutical therapy.

A targeting ligand coupled to a potent toxin is an attractive antitumor agent, but the application of the targeted ligand to clinical therapy has been limited because of its failure to concentrate at the site of the tumor (Shockley et al., 1992; Kreitman, 1999). The injection of agents directly into the tumor may circumvent these problems because therapy can be concentrated at the site of the tumor, thereby diminishing the risk to nontarget organs. The leakage of the agents from tumors into systemic flow may cause injury to normal tissues. If the claudin-4-targeting molecule leaks from the injected tumor tissue into the systemic flow, the leaked molecule interacts with tissues on the apical side. We found that C-CPE-PSIF is cytotoxic in a cellular polarity-dependent manner (Fig. 3, E and F). Singh et al. (2001) also showed that Caco-2 cells are more sensitive to CPE when CPE is applied to the basal side rather than the apical side (Singh et al., 2001). These findings indicate that preparation of high affinity and specificity of claudin-4 binder may be critical for clinical application of a claudin-4-targeting ligand such as C-CPE-PSIF.

In summary, we prepared a novel claudin-4-targeting molecule, C-CPE-PSIF, which consists of C-CPE$_{194-319}$ and PSIF, and we found that the cytotoxicity of C-CPE-PSIF is specific to the expression and localization of claudin-4 and that the intratumoral administration of C-CPE-PSIF suppressed tumor growth. This is the first report that a claudin-4-targeting ligand C-CPE is useful for antitumor therapy. Future improvements in the cytotoxicity of the claudin-4-targeting strategy will be useful for its clinical application.

Acknowledgments

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TOPICS

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生体バリアを利用した創薬研究

第1回日本DDS学会奨励賞(臨床)受賞によせて

Claudin binder as a novel drug delivery system : the 1st Encouragement Award in the 25th Annual Meeting of the Japan Society of Drug Delivery System

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Epithelium surrounds the body separating intra and outer body. Passing across epithelium is the first step of drug absorption. Most malignant tumors are derived from epithelium. Moreover, epithelium is the first line of defense against pathological microorganisms. These findings strongly indicate that epithelium is a promising target for drug discovery. Claudin family, which is identified by Dr Tsukita group, is a key molecule for epithelial barrier.

In this review, we discussed about claudin as a potential target for drug development.

進化の過程において，多細胞生物は生体内外および組織内外を隔てる障壁として上皮細胞層を発達させてきた．上皮細胞層の透過過程が薬物吸収の第一ステップであること，悪性腫瘍の90%が上皮由来であること，上皮細胞層は感染性病原微生物の侵入門戸になっていることから，上皮細胞は創薬ターゲットとして有用な性質を有している．1993年以降，上皮細胞バリア構成蛋白質occludin，claudinなどが同定され，生体バリアの分子基盤が詳らかにされつつある．

本総説では，当研究グループで推進している生体バリアの分子基盤claudinを利用した創薬研究について，その一端を紹介したい．

60兆個の細胞からなる個体は上皮細胞層により生体内外に区別され，さらに生体内では上皮細胞層および血管内皮細胞層によって脳，血管，腎臓などのコンパートメントが形成されている．コンパートメント内の恒常性を維持するためには内外の物質移動を制御することが不可欠であり，上皮細胞層や血管内皮細胞層は，生体内外および組織内外を隔てるバリアとして機能することにより，恒常性維持に深く関わっている．これらの細胞層では，隣接する細胞間にtight junction(TJ)が発達しており，TJによって細胞間隙はシールされ物質の漏れが抑制されている．

70年代にはTJがストランド様の構造をしていることが見いだされていたものの，TJ分子基盤は長年にわたり不明なままであった[1]．93年，京大月田承一郎博士のグループによりTJ構成蛋白質としてoccludinが同定されTJが蛋白質で構成されていることがはじめて明らかにされた[2]．さらに98年に，同グループによりTJバリアの機能本体としてclaudinが同定された[3]．

Claudinは24種の分子からなるfamilyを形成しており，細胞間隙におけるホモフィリック/ヘテロフィリックな結合によりTJをシールしていると考えられている[4,5]．興味深いことにバリア機能には組織特異性が認められ，claudin-1は皮膚バリア，claudin-5は血液脳関門バリア，claudin-11は血液精巣関門バリアを担っており，claudinを利用した新規薬物送達法開発の可能性が強く示唆されている[6~8]．また，悪性腫瘍の90%を占める上皮がんでは多くのがん種でclaudinの発現異常が観察されており，claudinは上皮がんの創薬ターゲットとしても衆目を集めている[9,10]．さらに，claudinが粘膜

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表1　タイトジャンクション研究

年代	研究内容
1973年	TJストランドの発見
1982年	脂質ミセル説の提唱
1993年	Occludinの発見
1998年	Claudinの発見
1999年以降	Claudinバリア機能の証明
2005年	Tricellulinの発見

表2　既存の生体バリアを利用した創薬研究

吸収促進剤	生体内標的分子
EDTA	Ca^{2+}のキレート
オレイン酸	脂質二重膜の流動性亢進
NO供与剤	不明
カプリン酸ナトリウム	ホスホリパーゼC

免疫組織で高発現していること[11]，occludinおよびclaudinがC型肝炎ウイルスの感染受容体であることも報告されており[12,13]，TJ分子基盤を利用した粘膜ワクチンおよび感染阻害薬創出の可能性も見いだされている．

このように，月田グループによるoccludinおよびclaudinの発見に端を発した上皮細胞バリア研究の進展に伴い，従来までの「上皮細胞バリア＝物質透過障壁」という概念に加え，「上皮細胞バリア分子基盤＝創薬ターゲット」という新たな萌芽が生まれつつある．

本稿では，上述した本邦発の新規創薬ターゲット，上皮細胞バリア制御分子claudinの可能性について，薬物送達法および抗がん剤開発に焦点を絞り，当研究グループの研究成果を概説する．

上皮細胞バリアの分子基盤

隣接する細胞は10～20 nmの空隙を隔てて向かい合っており，アピカル側では隣接する細胞膜がストランド様の構造体TJによって密着し，細胞間の距離がゼロとなるキッシングポイントにより細胞間隙における物質の移動が制御されている[1]．電子顕微鏡を用いた解析から，TJストランドは網目状にラテラル面に広がり物質の移動を制御していることが1970年代には明らかにされていたものの，このストランドの本体については脂質ミセル説なども提唱され長年にわたり議論が続いていた(表1)[14]．

1993年に，京大月田グループにより，はじめてのTJ構成蛋白質としてoccludinが見いだされ，TJストランドが蛋白質で構成されていることが示された[2]．1998年に新たなTJ構成蛋白質としてclaudinが同定され，1999年以降claudinがTJバリア能の本体を担うことを示唆する知見が集積しつつある[3,15,16]．さらに2005年には，同グループにより，上皮細胞層において三つの細胞が交わるジャンクション(トリセルラージャンクション)の構成蛋白質としてtricellulinが同定されている[17]．Tricellulinをノックダウンすると上皮細胞バリア機能が低下することから，上皮細胞バリアは2細胞間のシール機構と3細胞間のシール機構により構成されていることが実験的に証明された[17]．

上皮細胞バリアを利用した第一世代の創薬研究

前述したように，上皮細胞層は生体内外を隔てる障壁として機能しており，薬物吸収に際しては上皮細胞層の透過が不可欠となっている．すでに30年あまりにわたり上皮細胞バリアを利用した創薬研究として吸収促進剤が研究されてきており，EDTA，オレイン酸，NO供与剤，カプリン酸ナトリウムなどが吸収促進活性物質として見いだされている(表2)．それぞれの作用点は細胞間隙に存在するカルシウムイオン，細胞膜，phospholipase Cなどであり，TJの開口を通じて吸収促進効果を発揮すると考えられている．

これらアプローチが研究開発されていた1980年～98年にはTJ構成蛋白質は未解明であり，吸収促進作用に組織特異性が乏しいこと，TJの開口に伴い薬物以外の物質の非特異的な流入が生じることなどから，臨床応用されている吸収促進剤はカプリン酸ナトリウムなどにすぎない[18～20]．

上皮細胞バリアを利用した第二世代の創薬研究

当研究グループでは，上述した吸収促進剤の有する課題はTJを利用した薬物送達法の限界を示しているのではなく，TJの分子基盤に立脚したア

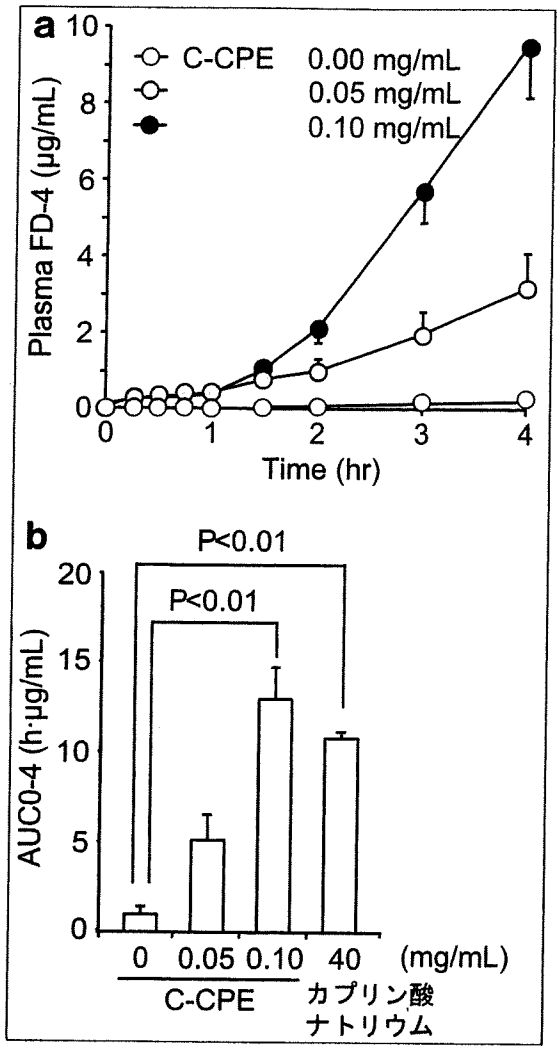

図1 C-CPEの腸管吸収促進活性

a：血漿中FD-4濃度の経時変化，**b**：AUC値
FITCラベルした分子量4000のデキストラン(FD-4)をモデル薬物として用いて，ラット腸管ループ法によりC-CPEの吸収促進活性を解析(Kondoh M et al., 2005[25]を一部改変)．

プローチが採られてこなかったことに起因していると考え，上述した吸収促進剤を第一世代のTJ modulatorと定義し，TJの分子基盤に立脚した第二世代のTJ modulatorを利用した薬物送達研究を進めてきた[21]．

1993年，京大月田グループによりTJ構成蛋白質として4回膜貫通蛋白質occludinが見いだされ，TJが蛋白質によって構成されていることがはじめて明らかにされた[2]．しかしながら，occludinを欠損させても，機能的，構造的に正常なTJが構成されていたことから，occludinはTJバリア機能分子ではないことが示唆された[22]．この報告とほぼ時を同じくして，1998年に新たなTJ構成蛋白質claudinが同定された(**表1**)[3]．

Claudinは分子量23 kDaの4回膜貫通蛋白質であり，現在までに24種類の分子が見いだされている．非常に興味深いことに，発現およびバリア機能には組織特異性が認められ，claudin-1欠損マウスでは重層上皮細胞のバリア機能が異常をきたし，分子量600程度の分子が皮膚を透過し，claudin-5欠損マウスでは血液脳関門を分子量800程度の分子が通過する[5,6,8,15,23]．さらに，claudin-11は血液精巣関門バリアを担っていることも明らかにされている[7]．これらの報告はclaudinを分子特異的に制御することができれば新たな薬物送達法の開発に繋がることを意味している．

現在のところ，claudinのバリア機能を阻害する分子としてはウエルシュ菌エンテロトキシン(CPE)のC末断片(C-CPE)のみがclaudin-4のバリア機能を阻害する分子として知られている[16,24]．そこでC-CPEをclaudin modulatorのモデル分子として用いて，claudinを利用した薬物送達法創出の可能性について検証を試みた．

まず，C-CPEの薬物送達活性を分子量4000のデキストラン(FD-4)をモデル分子として用いてラット腸管ループ法により解析したところ，C-CPE添加濃度依存的に血漿中FD-4濃度は上昇していた(**図1a**)．C-CPEの吸収促進活性は臨床応用されている吸収促進剤であるカプリン酸ナトリウムに比して400倍もの値を示しており(**図1b**)，さらに吸収促進効果には組織特異性も観察されていた[25]．

CPEはC末30アミノ酸を介して受容体と相互作用することが示唆されている[26]．そこで，C-CPEの薬物送達活性におけるclaudin-4の関与を検討するために，受容体結合領域をすべて欠損させたC-CPE289およびその一部を欠損させたC-CPE303を作成し，ラット腸管における吸収促進活性を解析したところ，C-CPE289，C-CPE303処理では吸収促進活性が著しく減弱していた(**図2a, b**)．

以上の結果より，claudinを利用した粘膜吸収促進法の有用性が示唆される．

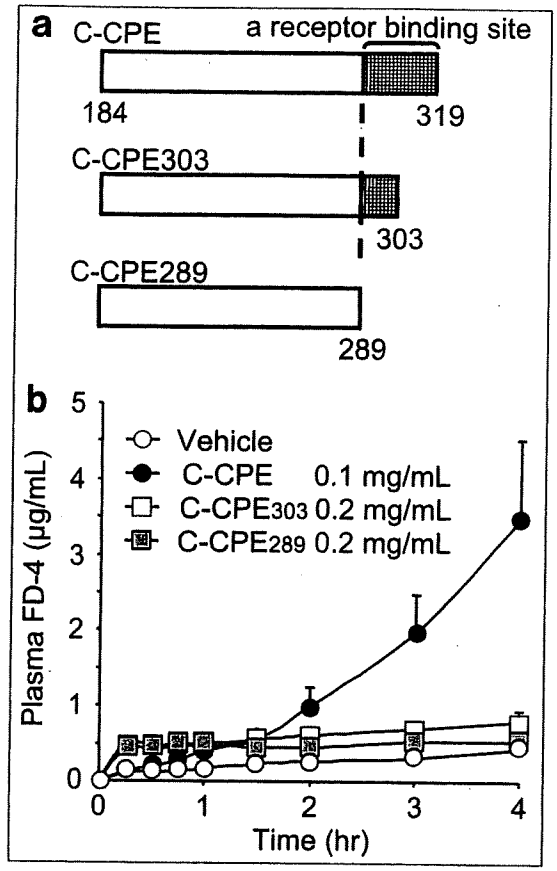

図2 C-CPEの吸収促進作用におけるclaudin-4の関与

a：C-CPEs. b：腸管吸収促進効果
C-CPEのclaudin-4結合領域を欠損させた変異体(a)のFD-4吸収促進活性を解析(b)．なお，データはKondoh M et al., 2005[25]を一部改変．

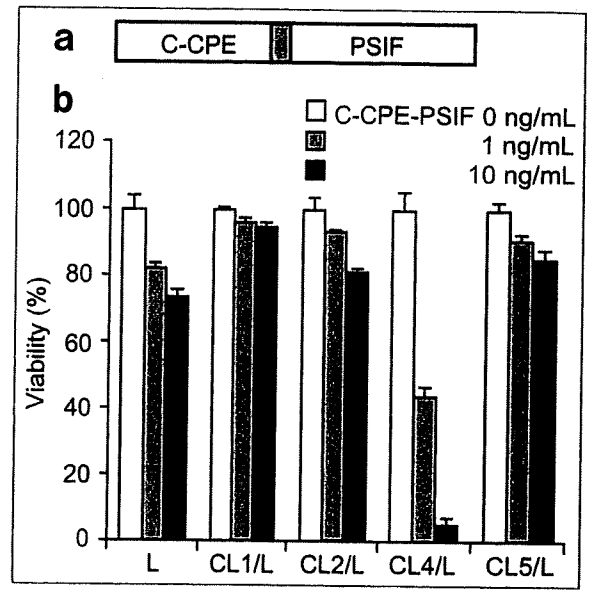

図3 Claudin-4指向性分子

a：C-CPE-PSIF. b：Claudin(CL)特異性
Pseudomonas exotoxinの蛋白質合成阻害ドメイン(PSIF)とC-CPEとの融合体(a)を作製し，各種claudin発現細胞に各濃度24時間処理後にWSTアッセイにより細胞毒性を解析(b)．なお，データはSaeki R et al., in press[26]を一部改変．

上皮細胞バリアを利用した第三世代の創薬研究

上述したように，従来の生体バリアを利用した創薬研究は薬物吸収促進に焦点が当てられてきた．2000年以降，悪性腫瘍(年間死者数700万人)の90%を占める上皮由来のがんとclaudinとの関連性についても，多方面からの研究が進み，卵巣がん，膵臓がん，膀胱がんなど12種類あまりのがんにおけるclaudinの高発現が見いだされ，claudinががんターゲティングの標的分子としても注目されている[9,10]．

さて，正常な上皮細胞層では水平方向に細胞は分裂しコンタクトインヒビッションにより細胞増殖は制御されている．一方，上皮細胞ががん化すると細胞分裂軸が回転し垂直方向への分裂がはじまり，コンタクトインヒビッションがかからず腫瘍組織を形成していくと考えられている．このがん化早期イベントである分裂軸の回転を利用したがん治療戦略を構築することができれば，がんの早期診断・早期治療法の開発に繋がるといえる．

当研究グループでは，分裂軸の回転に伴いlateral面からapical面に露出するTJ構成蛋白質claudinを利用した新規がん治療法の開発を目指し，claudin-4 binderを利用したがん治療戦略の構築を試みている[27,28]．

まず，claudin指向性抗がん剤のモデルとして，claudin-4結合分子C-CPEと緑膿菌由来の蛋白質合成阻害因子(PSIF)との融合蛋白質を作製した(図3a)．C-CPE-PSIFはclaudin-1, -2, -5発現L細胞では10 ng/mLでもまったく細胞障害性を示さないのに対して，claudin-4発現L細胞では1 ng/mL処理でも顕著な細胞毒性を発揮していた(図3b)．さらに，Caco-2細胞の単層膜培養系にapical側，もしくはbasal側からC-CPE-PSIFを添加したとこ

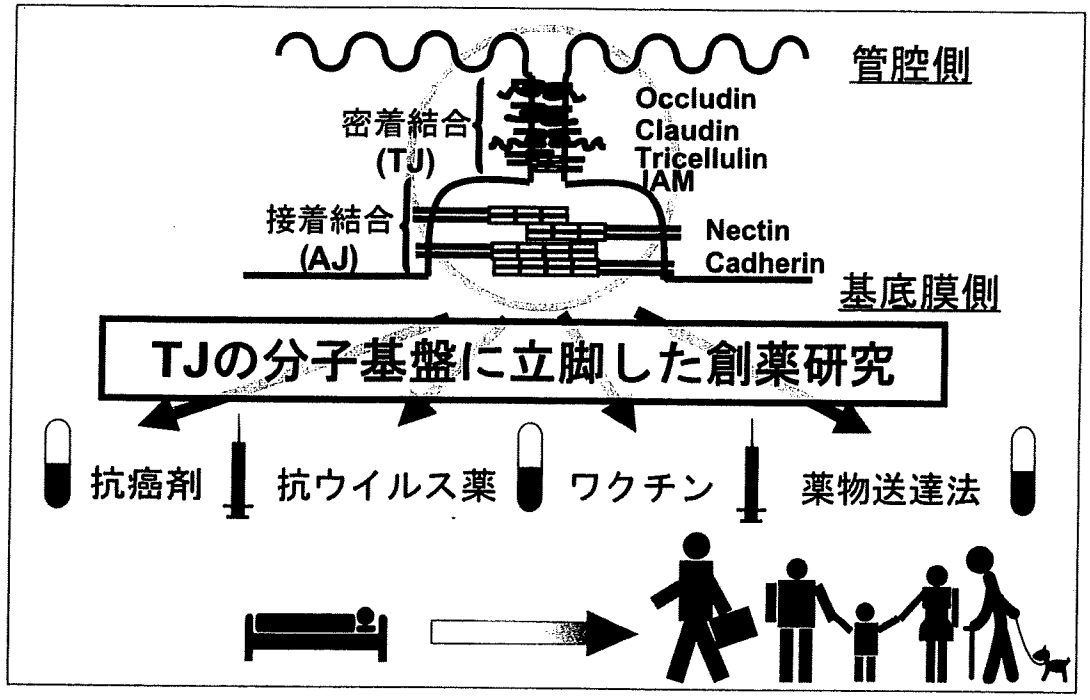

図4　生体バリアを利用した創薬研究

ろ，C-CPE-PSIF を basal 側から加えたときのみ細胞毒性が観察され，C-CPE は claudin の局在性を認識する新しいタイプの抗がん活性分子であると推察された(data not shown)．さらに，マウス乳がん由来細胞株 4T1 細胞を用いて C-CPE-PSIF の抗腫瘍効果を解析したところ，C-CPE-PSIF 処理で *in vitro* および *in vivo* での抗腫瘍活性が認められ体重減少などの副作用は観察されず，claudin-4 結合性を消失させた変異体では *in vitro*，および *in vivo* 抗腫瘍活性がともに消失していた[28]．

これらの結果を踏まえ，現在当研究グループでは，がん化の早期イベント「分裂軸の回転」を標的とした新規がん診断法・治療法の開発を目指し，新規 claudin binder の創出，既存の claudin binder のプローブ化，最適化などを進めている．

まとめ

今回は紙面の都合上割愛させていただいているが，粘膜免疫組織に高発現している claudin を利用したワクチン開発，ウイルスの感染受容体である claudin-1 や occludin を標的とした感染阻害薬の開発などに関しても研究が進展しつつあり，現在までに claudin を利用した非侵襲性投与技術，claudin 指向性抗がん剤，claudin 指向性粘膜ワクチンの創製に成功し，世界に先駆けて生体バリアの分子基盤を利用した創薬戦略の有用性を提唱してきた[19,21,25,28~30]．

周知のように，わが国にはカドヘリンをはじめとした上皮細胞生物学の基礎的知見が集積している．当研究グループでは，本邦固有の上皮細胞生物学の土壌に生まれた創薬研究の萌芽を育み，わが国独自の生体バリアを利用した創薬研究領域を開拓し，本邦発の創薬シーズを一つでも多く "bench side to bed side" へと顕在化できるよう最善を尽くしていきたい(図4)．

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A claudin-4 modulator enhances the mucosal absorption of a biologically active peptide

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ABSTRACT

Biologics, such as peptides, proteins and nucleic acids, are emerging pharmaceuticals. Passage across the epithelium is the first step in the absorption of biologics. Tight junctions (TJ) function as seals between adjacent epithelial cells, preventing free movement of solutes across the epithelium. We previously found that modulation of a key TJ component, claudin-4, is a potent method to enhance jejunal absorption when we used dextran as a model drug and the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) as a claudin-4 modulator. Here, we investigated whether the claudin-4 modulator enhances jejunal, nasal and pulmonary absorption of a biologics human parathyroid hormone derivative, hPTH(1-34). The claudin-4 modulator enhanced nasal but not jejunal and pulmonary absorption of hPTH(1-34). C-CPE is hydrophobic with low solubility of less than 0.3 mg/ml, but deletion of 10 amino acids at the N-terminal of C-CPE increased its solubility by 30-fold. Moreover, the N-terminal truncated C-CPE bound to claudin-4, modulated the TJ-barrier and enhanced jejunal absorption of dextran. The N-terminal-truncated C-CPE also enhanced jejunal and pulmonary absorption of hPTH(1-34). This report is the first to indicate that a claudin-4 modulator may be a promising enhancer of the jejunal, pulmonary and nasal absorption of a peptide drug.

1. Introduction

Recent progress in genomic and proteomic technologies has yielded some new biologics, such as peptides, proteins and nucleic acids, as pharmaceutical candidates. Passage across the mucosal epithelium of the intestine, nose and lung is the first step in drug absorption. However, most biologics are hydrophilic molecules that are poorly absorbed by the mucosa. Although injection is a compelling route for the administration of biologics, a transmucosal delivery system would be an ideal administration route for biologics because it is noninvasive and therefore would provide a higher quality of life to patients. However, it is difficult to develop a transmucosal delivery system since the epithelium plays a pivotal role in the barrier separating the inside of the body from the outside environment.

Tight junctions (TJ) exist between adjacent epithelial cells and seal the paracellular space, preventing free movement of solutes [1]. To facilitate drug absorption, modulators of the epithelial barrier have been investigated since 1960s [2,3]. Many TJ modulators, such as fatty acids, bile salts, a polysaccharide and a toxin fragment, have been developed [4–6]. However, the biochemical structures of TJs remained uncharacterized until 1998, and a drug absorption enhancer based on TJ-components has never been fully developed [7]. Occludin, a 65-kDa tetra-transmembrane protein, was the first TJ-structural component to be identified [8]. Claudin, a 23-kDa integral membrane protein bearing tetra-transmembrane domains, is the functional component of the TJ-barrier [9,10]. The claudin family consists of 24

Abbreviations: TJ, tight junction; C-CPE, the C-terminal fragment of *Clostridium perfringens* enterotoxin; C-CPE184, C-terminal fragment of *Clostridium perfringens* enterotoxin from 184 to 319 amino acids; hPTH, human parathyroid hormone; CPE, *Clostridium perfringens* enterotoxin; DDM, n-Dodecyl-β-D-maltoside; EDC, N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide; NHS, N-hydroxysuccinimide; C-CPE194, C-terminal fragment of *Clostridium perfringens* enterotoxin from 194 to 319 amino acids; C-CPE205, C-terminal fragment of *Clostridium perfringens* enterotoxin from 205 to 319 amino acids; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BV, budded baculovirus; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; TEER, transepithelial electric resistance; FD-4, fluorescein isothiocyanate-dextran with a molecular weight of 4 kDa; AUC, the area under the plasma concentration; BA, bioavailability.

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members with different barrier functions among tissues. For example, claudin-1 and -5 function in the epidermal barrier and blood-brain barrier, respectively [11,12]. Claudin is heterogeneously expressed in epithelial cells of the gut, lung and nose [13–15]. The extracellular loop domains of claudin between adjacent cells are estimated to be involved in the paracellular tightness of the cleft between cells, resulting in prevention of solute movement by the formation of TJs [16,17]. Therefore, a molecule that can bind to the extracellular loop domain of claudin may be a novel type of mucosal-absorption enhancer.

The 35-kDa *Clostridium perfringens* enterotoxin (CPE) causes food poisoning in humans [18]. The receptor for CPE is claudin-4 [10]. The C-terminal fragment of CPE (amino acids 184–319; C-CPE184) is the receptor-binding region of CPE. Treatment of cells with C-CPE184 disrupted the TJ-barrier through its interaction with the second extracellular loop of claudin-4, indicating that C-CPE184 is a claudin-4 modulator [10,19,20]. We previously found that C-CPE184 enhanced the jejunal absorption of dextran (molecular mass, 4 kDa) over 400-fold compared with a clinically used absorption enhancer, sodium caprate, and that deletion of the claudin-4-binding region in C-CPE attenuated the absorption-enhancing effect of C-CPE [21]. Claudin-4 is also expressed in nasal and pulmonary epithelial cells [14,15,22]. Thus, a claudin-4 modulator may be a promising candidate for the enhancement of not only jejunal absorption but also pulmonary and nasal absorption of biologics; however, the ability of a claudin modulator to enhance the mucosal absorption of biologics has not yet been investigated.

In the present study, we investigated whether claudin-4 modulation enhanced jejunal, nasal and pulmonary absorption of a peptide drug, human parathyroid hormone derivative (hPTH(1-34)) using C-CPE184 and its derivatives. We found that a claudin-4 modulator was a novel mucosal-absorption enhancer of a peptide drug.

2. Materials and methods

2.1. Materials

Human parathyroid hormone derivative (hPTH(1-34)) was prepared as described previously [23]. n-Dodecyl-β-D-maltoside (DDM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Anti-claudin antibodies and anti-his-tag antibody were obtained from Invitrogen (Carlsbad, CA) and EMD Chemicals Inc. (Darmstadt, Germany), respectively. CM5 sensor chips, amine-coupling reagents (N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), and ethanolamine-HCl) and HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P20) were obtained from GE Healthcare (Buckinghamshire, UK). All reagents used were of research grade.

2.2. Preparation of N-terminal-truncated C-CPE derivatives

We prepared expression vectors of N-terminal region-truncated C-CPE184 (amino acids 184–319). These vectors expressed amino acids 194–319 (C-CPE194), 205–319 (C-CPE205), 212–319 (C-CPE212), 219–319 (C-CPE219) and 224–319 (C-CPE224). Insert fragments of each C-CPE mutant were amplified from C-CPE184 cDNA (kindly provided by Dr. Y. Horiguchi, Osaka University) by polymerase chain reaction with forward primers (5′-ATGCTCGAGGATATAGAAAAAGAAATCCTT-3′ for C-CPE194, 5′-ATGCTCGAGGCTACAGAAAGATTAAATTTAACTG-3′ for C-CPE205, 5′-ATGGGGGAGGCTACAGAAAGATTAAATTTAACTG-3′ for C-CPE212, 5′-ATGCTCGAGGCTGAAGCCCAAGATTAAATTTAACTG-3′ for C-CPE219, 5′-ATGCTCGATCTATGAAGATTAAATTTAACTG-3′ for C-CPE224) and a common reverse primer (5′-TTTGCTAGCTAAGATTCTATATTTTTGTCC-3′). The resultant C-CPE fragments were cloned into the pET16b vector. The plasmids were transduced into *E. coli* BL21 (DE3), and protein expression was stimulated by the addition of isopropyl-1-thio-β-D-galactoside. The cell lysates were applied to HisTrap™ HP (GE Healthcare), and C-CPEs were eluted with imidazole. The solvent was exchanged with phosphate-buffered saline (PBS) 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 (SDS-PAGE) followed by staining with Coomassie Brilliant Blue. C-CPEs were quantified by using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL) with bovine serum albumin as a standard.

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

Claudin-displaying BV was prepared as described previously [24]. Briefly, mouse claudin-1 and -4 cDNA fragments were cloned into the baculoviral transfer vector pFastBac1 (Invitrogen). Recombinant baculoviruses were generated by using the Bac-to-Bac system according to the manufacturer's instructions (Invitrogen). Sf9 cells were cultured in Grace's Insect medium (Invitrogen) containing 10% FBS at 27 °C and infected with the recombinant baculovirus. Seventy-two hours after infection, the BV fraction was isolated from the culture supernatant of the infected Sf9 cells by centrifugation at 40,000 × *g* for 25 min. The pellets of the BV fraction were suspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MI) and then stored at 4 °C. The expression of claudins in the BV fraction was confirmed by SDS-PAGE and immunoblot with antibodies against claudins.

2.4. Enzyme-linked immunosorbent assay (ELISA) with claudin-displaying BV

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

2.5. Preparation of recombinant claudin-4 protein

Recombinant claudin-4 protein was prepared by using Sf9 cells infected with recombinant baculovirus, as previously reported [25,26]. Briefly, the C-terminal his-tagged claudin-4 cDNA fragment was cloned into pFastBac1, and recombinant baculovirus was generated by using the Bac-to-Bac baculovirus expression system. Sf9 cells were infected with the recombinant baculovirus. After 52–56 h of infection, the cells were harvested by centrifugation. The cells were washed with PBS and were resuspended in 10 mM Hepes, pH 7.4, 120 mM NaCl with protease inhibitor tablets (Complete Mini, EDTA-free, Roche Applied Science (Indianapolis, IN)), 1 mM phenylmethylsulfonyl fluoride and 20 units/ml DNase I. The cells were lysed by the addition of 2% of DDM and were then centrifuged. The resultant supernatant was applied to HisTrap™ HP, and claudin-4 was eluted with imidazole. The solvent for claudin-4 was exchanged to PBS containing 0.2% DDM by gel

filtration with a HiTrap Desalting column (GE Healthcare). Purification of claudin-4 was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue.

2.6. Surface plasmon resonance (SPR) analysis

SPR 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 μl/min. Claudin-4 was diluted to 2.5 μg/ml in 10 mM MES buffer (pH 6.5) and injected for 2 min over the surface at a flow rate of 10 μl/min. Excess activated groups were blocked by a 5-min injection of 1 M ethanolamine (pH 8.5) at a flow rate of 10 μl/min. Approximately 1000 RU of claudin-4 was immobilized by using this protocol. Single-cycle kinetics experiments were performed at 25 °C with a flow rate of 30 μl/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 1×10^5 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 μg/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 μg) 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 [^{125}I-Tyr34] hPTH(1-34) (15,000–20,000 cpm/100 μl) 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 (μg/kg))/(AUC (iv)(ng·min/ml)/dose (iv) (μg/kg)). AUC (iv) indicates the $AUC_{0-120\ min}$ of intravenously administered hPTH(1-34) (10 μg/kg), and the AUC value is 208.6 ± 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 ($AUC_{0-6\ h}$) 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 μg 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 μl. 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 μg 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