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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 its 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 CytoTox-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-

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).

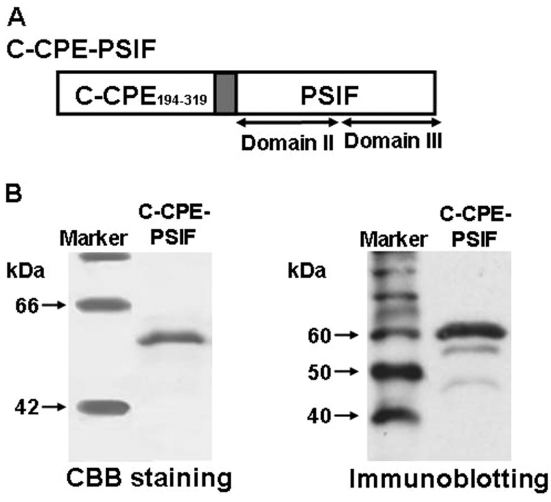

Fig. 1. Preparation of C-CPE-PSIF. A, schematic structure of C-CPE-PSIF. C-CPE-PSIF is a fusion protein of C-CPE and PSIF. C-CPE is the C-terminal fragment of CPE 194 to 319 aa (Van Itallie et al., 2008). The dark area indicates the putative receptor-binding region of C-CPE located in its C terminus (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-

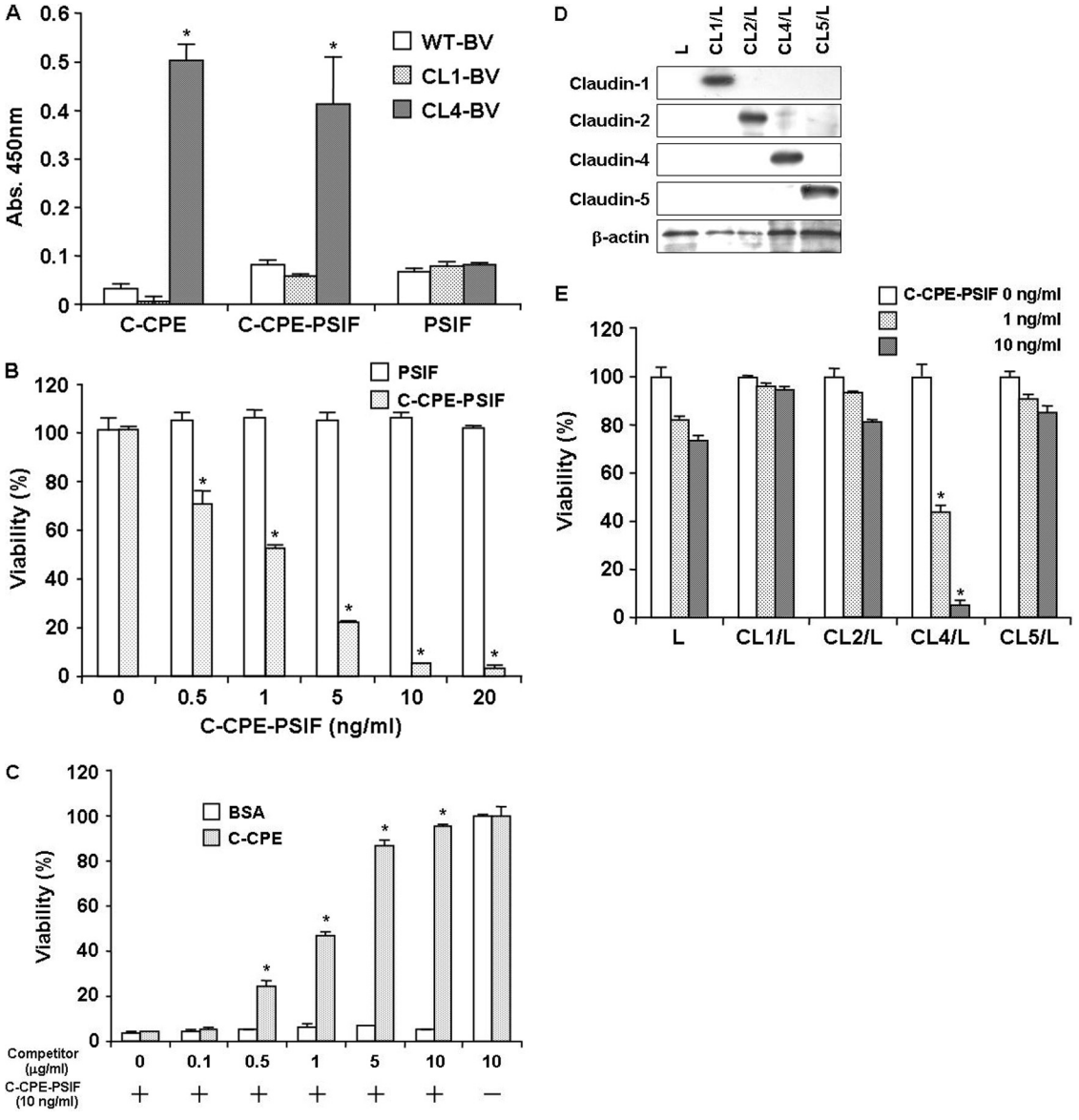

Fig. 2. Characterization of C-CPE-PSIF. A, interaction of C-CPE-PSIF with claudins. Wild-type BV (WT-BV), claudin-1-BV (CL1-BV), or claudin-4-BV (CL4-BV) was adsorbed onto a 96-well immunoplate at 0.5 μg/well. Then, 7.5 μmol C-CPE, C-CPE-PSIF, or PSIF was added to the well, and the protein bound to the 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 ($p < 0.01$).

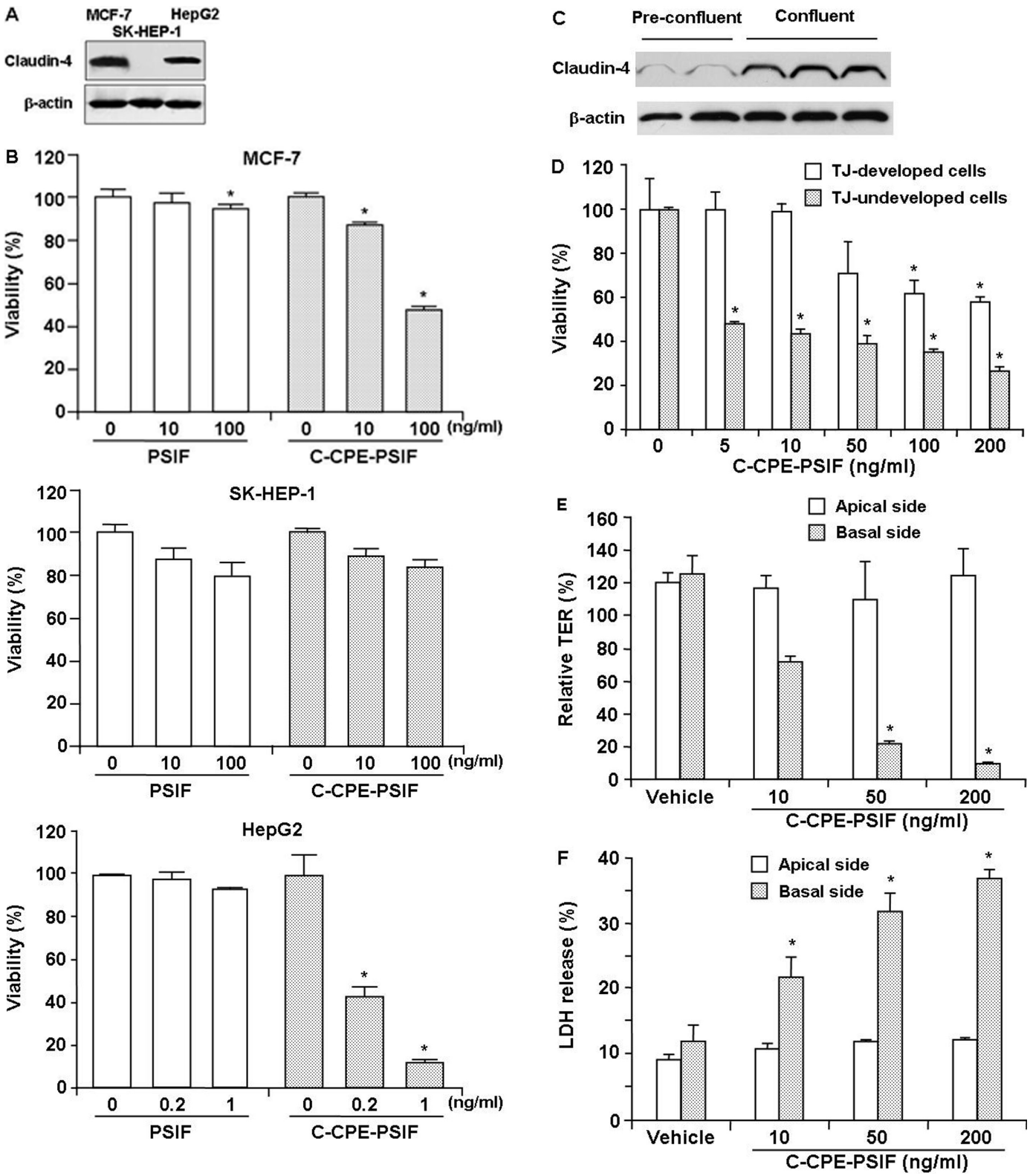

Fig. 3. Cytotoxic specificity of C-CPE-PSIF. A, expression of claudin-4 in SK-HEP-1 and HepG2 cells. The cell lysates were subjected to SDS-PAGE, followed by Western blotting with anti-claudin-4 Ab. MCF-7 cells were used as a positive control. B, cytotoxicity of C-CPE-PSIF in SK-HEP-1 and HepG2 cells. 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

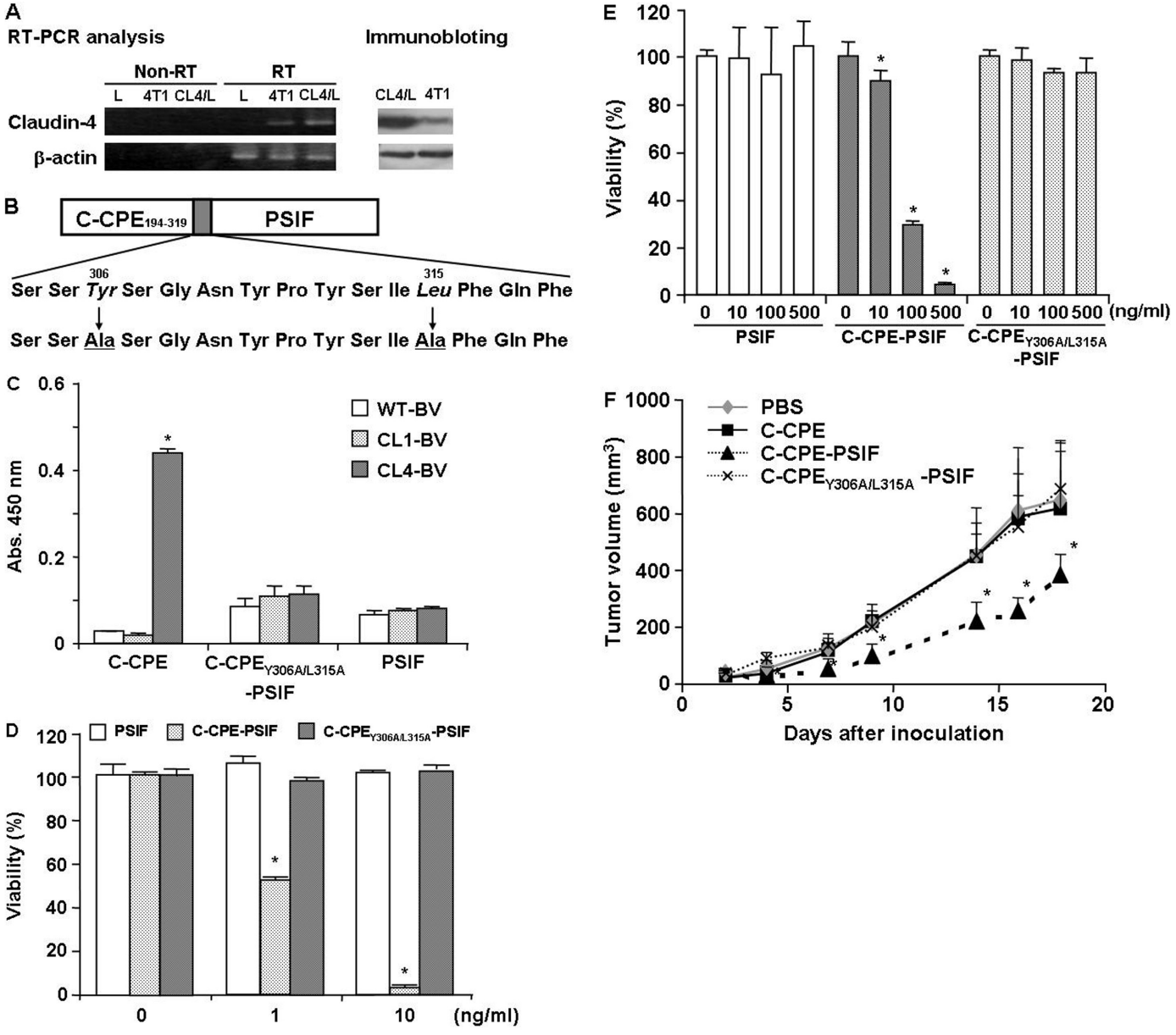

Fig. 4. In vivo antitumor activity of C-CPE-PSIF. A, expression of claudin-4 in 4T1 cells. Total RNA was extracted from the cells, followed by reverse transcription-polymerase chain reaction analysis (left). Cell lysates were subjected to SDS-PAGE, followed by Western blotting (right). β-Actin is an 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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References

Allen TM and Cullis PR (2004) Drug delivery systems: entering the mainstream. *Science* **303:**1818–1822.
Anderson JM and Van Itallie CM (1995) Tight junctions and the molecular basis for regulation of paracellular permeability. *Am J Physiol* **269:**G467–G475.
Deckert PM (2009) Current constructs and targets in clinical development for antibody-based cancer therapy. *Curr Drug Targets* **10:**158–175.
Dunham W (2007) Report sees 7.6 million global 2007 cancer deaths. 2007 Dec 17, Reuters, New York. Available at http://www.reuters.com/article/healthNews/idUSN1633064920071217.
Ebihara C, Kondoh M, Hasuike N, Harada M, Mizuguchi H, Horiguchi Y, Fujii M, and Watanabe Y (2006) Preparation of a claudin-targeting molecule using a C-terminal fragment of Clostridium perfringens enterotoxin. *J Pharmacol Exp Ther* **316:**255–260.
Fujita K, Katahira J, Horiguchi Y, Sonoda N, Furuse M, and Tsukita S (2000) Clostridium perfringens enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein. *FEBS Lett* **476:**258–261.
Furuse M and Tsukita S (2006) Claudins in occluding junctions of humans and flies. *Trends Cell Biol* **16:**181–188.
Hanna PC, Wieckowski EU, Mietzner TA, and McClane BA (1992) Mapping of functional regions of *Clostridium perfringens* type A enterotoxin. *Infect Immun* **60:**2110–2114.
Jain RK (1987) Transport of molecules in the tumor interstitium: a review. *Cancer Res* **47:**3039–3051.
Jain RK (1989) Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J Natl Cancer Inst* **81:**570–576.
Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, and Thun MJ (2008) Cancer statistics, 2008. *CA Cancer J Clin* **58:**71–96.
Katahira J, Inoue N, Horiguchi Y, Matsuda M, and Sugimoto N (1997) Molecular cloning and functional characterization of the receptor for Clostridium perfringens enterotoxin. *J Cell Biol* **136:**1239–1247.
Kominsky SL (2006) Claudins: emerging targets for cancer therapy. *Expert Rev Mol Med* **8:**1–11.
Kominsky SL, Vali M, Korz D, Gabig TG, Weitzman SA, Argani P, and Sukumar S (2004) Clostridium perfringens enterotoxin elicits rapid and specific cytolysis of breast carcinoma cells mediated through tight junction proteins claudin 3 and 4. *Am J Pathol* **164:**1627–1633.
Kondoh M, Masuyama A, Takahashi A, Asano N, Mizuguchi H, Koizumi N, Fujii M, Hayakawa T, Horiguchi Y, and Watanbe Y (2005) A novel strategy for the enhancement of drug absorption using a claudin modulator. *Mol Pharmacol* **67:**749–756.
Kreitman RJ (1999) Immunotoxins in cancer therapy. *Curr Opin Immunol* **11:**570–578.
Kreitman RJ and Pastan I (2006) Immunotoxins in the treatment of hematologic malignancies. *Curr Drug Targets* **7:**1301–1311.
Long H, Crean CD, Lee WH, Cummings OW, and Gabig TG (2001) Expression of *Clostridium perfringens* enterotoxin receptors claudin-3 and claudin-4 in prostate cancer epithelium. *Cancer Res* **61:**7878–7881.
McClane BA and Chakrabarti G (2004) New insights into the cytotoxic mechanisms of *Clostridium perfringens* enterotoxin. *Anaerobe* **10:**107–114.
Meunier V, Bourrié M, Berger Y, and Fabre G (1995) The human intestinal epithelial cell line Caco-2; pharmacological and pharmacokinetic applications. *Cell Biol Toxicol* **11:**187–194.
Michl P, Buchholz M, Rolke M, Kunsch S, Löhr M, McClane B, Tsukita S, Leder G, Adler G, and Gress TM (2001) Claudin-4: a new target for pancreatic cancer treatment using *Clostridium perfringens* enterotoxin. *Gastroenterology* **121:**678–684.
Michl P and Gress TM (2004) Bacteria and bacterial toxins as therapeutic agents for solid tumors. *Curr Cancer Drug Targets* **4:**689–702.
Mitic LL and Anderson JM (1998) Molecular architecture of tight junctions. *Annu Rev Physiol* **60:**121–142.

Morin PJ (2005) Claudin proteins in human cancer: promising new targets for diagnosis and therapy. *Cancer Res* **65:**9603–9606.
Nichols LS, Ashfaq R, and Iacobuzio-Donahue CA (2004) Claudin 4 protein expression in primary and metastatic pancreatic cancer: support for use as a therapeutic target. *Am J Clin Pathol* **121:**226–230.
Ogata M, Chaudhary VK, Pastan I, and FitzGerald DJ (1990) Processing of *Pseudomonas* exotoxin by a cellular protease results in the generation of a 37,000-Da toxin fragment that is translocated to the cytosol. *J Biol Chem* **265:**20678–20685.
Paperna T, Peoples R, Wang YK, Kaplan P, and Francke U (1998) Genes for the CPE receptor (CPETR1) and the human homolog of RVP1 (CPETR2) are localized within the Williams-Beuren syndrome deletion. *Genomics* **54:**453–459.
Rangel LB, Agarwal R, D'Souza T, Pizer ES, Alò PL, Lancaster WD, Gregoire L, Schwartz DR, Cho KR, and Morin PJ (2003) Tight junction proteins claudin-3 and claudin-4 are frequently overexpressed in ovarian cancer but not in ovarian cystadenomas. *Clin Cancer Res* **9:**2567–2575.
Sakihama T, Sato T, Iwanari H, Kitamura T, Sakaguchi S, Kodama T, and Hamakubo T (2008) A simple detection method for low-affinity membrane protein interactions by baculoviral display. *PLoS One* **3:**e4024.
Santin AD, Cané S, Bellone S, Palmieri M, Siegel ER, Thomas M, Roman JJ, Burnett A, Cannon MJ, and Pecorelli S (2005) Treatment of chemotherapy-resistant human ovarian cancer xenografts in C.B-17/SCID mice by intraperitoneal administration of *Clostridium perfringens* enterotoxin. *Cancer Res* **65:**4334–4342.
Shockley TR, Lin K, Sung C, Nagy JA, Tompkins RG, Dedrick RL, Dvorak HF, and Yarmush ML (1992) A quantitative analysis of tumor specific monoclonal antibody uptake by human melanoma xenografts: effects of antibody immunological properties and tumor antigen expression levels. *Cancer Res* **52:**357–366.
Singh U, Mitic LL, Wieckowski EU, Anderson JM, and McClane BA (2001) Comparative biochemical and immunocytochemical studies reveal differences in the effects of *Clostridium perfringens* enterotoxin on polarized CaCo-2 cells versus Vero cells. *J Biol Chem* **276:**33402–33412.
Takahashi A, Komiya E, Kakutani H, Yoshida T, Fujii M, Horiguchi Y, Mizuguchi H, Tsutsumi Y, Tsunoda S, Koizumi N, et al. (2008) Domain mapping of a claudin-4 modulator, the C-terminal region of C-terminal fragment of *Clostridium perfringens* enterotoxin, by site-directed mutagenesis. *Biochem Pharmacol* **75:**1639–1648.
Takahashi A, Kondoh M, Masuyama A, Fujii M, Mizuguchi H, Horiguchi Y, and Watanabe Y (2005) Role of C-terminal regions of the C-terminal fragment of *Clostridium perfringens* enterotoxin in its interaction with claudin-4. *J Control Release* **108:**56–62.
Tsukita S and Furuse M (1999) Occludin and claudins in tight-junction strands: leading or supporting players? *Trends Cell Biol* **9:**268–273.
Tsukita S, Yamazaki Y, Katsuno T, Tamura A, and Tsukita S (2008) Tight junction-based epithelial microenvironment and cell proliferation. *Oncogene* **27:**6930–6938.
Van Itallie CM, Betts L, Smedley JG 3rd, McClane BA, and Anderson JM (2008) Structure of the claudin-binding domain of *Clostridium perfringens* enterotoxin. *J Biol Chem* **283:**268–274.
Vermeer PD, Einwalter LA, Moninger TO, Rokhlina T, Kern JA, Zabner J, and Welsh MJ (2003) Segregation of receptor and ligand regulates activation of epithelial growth factor receptor. *Nature* **422:**322–326.
Waldmann TA (1991) Monoclonal antibodies in diagnosis and therapy. *Science* **252:**1657–1662.
Wodarz A and Näthke I (2007) Cell polarity in development and cancer. *Nat Cell Biol* **9:**1016–1024.
Zumkeller W and Schofield PN (1995) Growth factors, cytokines and soluble forms of receptor molecules in cancer patients. *Anticancer Res* **15:**343–348.

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