A Claudin-Targeting Molecule as an Inhibitor of Tumor Metastasis

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ABSTRACT

Tumor metastasis of epithelium-derived tumors is the major cause of death from malignant tumors. Overexpression of claudin is observed frequently in malignant tumors. However, claudin-targeting antimetastasis therapy has never been investigated. We previously prepared a claudin-4-targeting antitumor molecule that consisted of the C-terminal fragment of Clostridium perfringens enterotoxin (C-CPE) fused to protein synthesis inhibitory factor (PSIF) derived from Pseudomonas exotoxin. In the present study, we investigated whether claudin CPE receptors can be a target for tumor metastasis by using the C-CPE-fused PSIF as a claudin-targeting agent. One of the most popular murine metastasis models is the lung metastasis of intravenously injected B16 cells. Therefore, we first investigated the effects of the C-CPE-fused PSIF on lung metastasis of claudin-4-expressing B16 (CL4-B16) cells. Intravenous administration of the C-CPE-fused PSIF suppressed lung metastasis of CL4-B16 cells but not B16 cells. Injection of C-CPE-fused PSIF also inhibited tumor growth and spontaneous lung metastasis of murine breast cancer 4T1 cells inoculated into the subcutis. Treatment with C-CPE-fused PSIF did not show apparent side effects in mice. These findings indicate that claudin targeting may be a novel strategy for inhibiting some tumor metastases.

Metastasis is the primary cause of death for most cancer patients (Gupta and Massagué, 2006; Steeg, 2006). Metastasis occurs during tumor growth and even during the surgical excision of the primary tumor. A great deal of effort has been made to overcome tumor metastasis, including the development of several potent methods for irradiation therapy, chemotherapy, and immunotherapy. However, 7 million patients worldwide die from malignant tumors each year, and the majority of malignant tumors are derived from the epithelium (Jemal et al., 2008). Thus, the development of a novel antitumor strategy against epithelium-derived cancer metastasis is needed.

The epithelium is located at the border between the outer and inner body and tissue. Spaces between the adjacent cells in epithelium are sealed by tight junctions (TJs). TJs prevent free movement of solutes across epithelium through the paracellular spaces and also maintain cellular polarity by regulating the localization of cellular membrane proteins, such as transporters, ion channels, and receptors, between the apical and basal sides of epithelial cells (Mitic and Anderson, 1998; Vermeer et al., 2003). Moreover, TJs control cell proliferation by regulating the localization of receptors on the cellular membrane and the intracellular signal transduction for cellular proliferation and differentiation (Vermeer et al., 2003; Matter et al., 2005). These TJ functions are frequently deregulated during tumorigenesis, and tumor cells often exhibit abnormalities in cellular polarity and differentiation (Martin and Jiang, 2001; Wodarz and Näthke, 2007). The loss of the integrity of the TJ seal in tumors may contribute to the supply of nutrition critical for tumor growth and the detachment of cancer cells from the primary tumor tissues, leading to the malignancy of tumors (Mullin, 1997; Martin and Jiang, 2001).

Freeze-fracture replica microscopy analysis revealed that TJs form a series of continuous strands within plasma membranes (Staehelin, 1973). TJ strands from the membranes of adjacent cells form a paired strand, and the paired strands seal the intercellular space between the adjacent cells. The TJ strands are composed of integral membrane proteins, ABBREVIATIONS: CPE, Clostridium perfringens enterotoxin; C-CPE, C-terminal fragment of CPE; PSIF, protein synthesis inhibitory factor; C-CPE-PSIF, C-CPE-fused PSIF; CL4-B16, claudin-4-expressing B16; TJ, tight junction; FBS, fetal bovine serum; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; ADR, adriamycin.

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such as occludin and claudin, and among them, claudin is a key structural and functional component of TJ seals (Furuse and Tsukita, 2006). Claudin, a tetra-transmembrane protein, comprises a family consisting of more than 20 members. The expression profiles and sealing functions of claudins differ among tissues. Claudin expression is often deregulated in human cancers such as breast, prostate, ovarian, gastric, and pancreatic cancers (Morin, 2005; Kominsky, 2006). There is a relationship between dysregulated claudin and metastasis (Agarwal et al., 2005; Dhawan et al., 2005). These findings indicate that claudin can be a potent target for cancer therapy.

*Clostridium perfringens* enterotoxin (CPE), a 35-kDa polypeptide, causes food poisoning in humans. CPE binds to its receptor, and then causes changes in the membrane permeability by complex formation on the plasma membrane followed by the induction of oncosis and apoptosis (McClane and Chakrabarti, 2004). The local administration of CPE suppresses solid tumor growth (Michl et al., 2001; Kominsky et al., 2004; Santin et al., 2005); however, whole CPE had been shown to suppress solid tumor growth (Saeki et al., 2009). In brief, pET-C-CPE-PSIF was transduced into *Escherichia coli* BL21 (DE3) (Novagen, Darmstadt, Germany), and the production of 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 MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol). The lysates were centrifuged, and the resultant supernatant was subjected to HitTrap Chelating HP (GE Healthcare). 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 BSA as a standard.

### Materials and Methods

**Cell Culture.** Mouse melanoma cell line B16-BL6 and mouse breast cancer cell line 4T1 were cultured in modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 10 mM HEPES, respectively. The cells were maintained in a 5% CO₂ atmosphere at 37°C.

**Preparation of B16 Cells Stably Expressing Claudin-4.** Mouse claudin-4 cDNA was subcloned into pcDNA3.1 plasmid coding a neomycin resistance gene. The claudin-4 expression vector was transfected into B16 cells, and B16 cells stably expressing claudin-4 (CL4-B16 cells) were isolated by Geneticin (G-418) selection.

**Immunoblot Analysis.** Cells were lysed in lysis buffer (50 mM Tris (pH 7.4), 8.25 mg/ml NaCl, 1% NP-40, 2 mM SDS, and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)). The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride membrane, followed by immunoblotting with anti-claudin-4 (Zymed Laboratories, South San Francisco, CA) or anti-β-actin Ab (Sigma-Aldrich). After incubation with a peroxidase-labeled secondary antibody (Millipore Bioscience Research Reagents, Temecula, CA), the immunoreactive band was visualized by chemiluminescence reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

**Cell Proliferation Assay.** B16 or CL4-B16-expressing cells (CL4-B16 cells) (2 × 10⁴ cells) were seeded into a 24-well plate. At the indicated time points, the cells were stained with trypan blue, and the number of viable cells was counted by using a hemocytometer under a microscope.

**In Vitro Metastasis Analysis.** A cell culture insert with an 8-μm pore size membrane (BD Biosciences Discovery Labware, Bedford, MA) was used for the invasion assay. The upper surface of the chamber was coated with 50 μl of 0.1 mg/ml Matrigel (BD Biosciences, San Jose, CA), and the lower chamber was filled with DMEM containing 10% FBS. Cells were cultured to approximately 80% confluence and serum-starved overnight (0.5% FBS). Then, cells (1 × 10⁶ cells) suspended in DMEM with 0.5% FBS were placed into the upper chamber and incubated at 37°C for 24 h. After incubation, noninvading cells were removed, and the membrane was stained with Diff-Quick reagent. The stained cells in five randomly selected fields were counted under a microscope.

**Preparation of C-CPE-PSIF.** The C-terminal fragment of CPE (C-CPE)-fused PSIF (C-CPE-PSIF) was prepared as described previously (Saeki et al., 2009). In brief, pET-C-CPE-PSIF was transduced into *Escherichia coli* BL21 (DE3) (Novagen, Darmstadt, Germany), and the production of 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 MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol). The lysates were centrifuged, and the resultant supernatant was subjected to HitTrap Chelating HP (GE Healthcare). 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 BSA as a standard.

**In Vitro Cytotoxic Analysis.** In the cytotoxic assay, B16 and CL4-B16 cells were seeded onto a 96-well culture dish at 1 × 10⁴ cells per well. After 24 h, the cells were treated with C-CPE-PSIF for 24 h at the indicated concentrations. The cytotoxicity was determined by using a WST-8 kit, according to the manufacturer’s instructions (Nacalai Tesque, Kyoto, Japan).

**In Vivo Antitumor Activity.** Female BALB/c mice (7−8 weeks old) and C57BL/6 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. The animal experiments were performed according to the guidelines of Osaka University. For an experimental metastasis model, B16 or CL4-B16 cells (5 × 10⁵ or 1 × 10⁶ cells) in 100 μl of PBS were injected intravenously into the tails of C57BL/6 mice. Then, mice received intravenously administered PBS or C-CPE-PSIF three times per week. Two weeks after the inoculation of the cells, the mice were sacrificed, and the number of lung metastasis colonies was counted under a microscope. For antitumor activity in B16 cells, 1 × 10⁵ B16 or CL4-B16 cells were injected subcutaneously into the right flanks of C57BL/6 mice. Vehicle or C-CPE-PSIF was intravenously administered three times per week. Calipers were used to measure the minimal and maximal tumor diameters, and the tumor volume was calculated as a × b × b/2, where a represents the maximal tumor diameter, and b represents the minimal tumor diameter. The body weights of mice were also monitored. For antitumor activity in 4T1 cells, 4T1 cells (1 × 10⁵ cells) in 50 μl of PBS were injected subcutaneously into the right flanks of BALB/c mice. PBS, C-CPE-PSIF, or adriamycin (ADR) was intravenously administered, and the tumor size and body weight of mice were monitored. Mice were sacrificed 35 days after tumor inoculation, and lung metastasis was evaluated by staining with India ink.

**Statistical Analysis.** Data were analyzed by using Dunnet’s method. The statistical significance for all comparisons was set at p < 0.05.

### Results

**Preparation of Claudin-4-Expressing B16 BL6 Cells.** To investigate the effects of C-CPE-PSIF on tumor metastasis, we selected murine B16 cells, which have a high propensity...
to metastasize to the lung (Saiki, 1997). Western blotting analysis revealed no expression of claudin-4 in B16 cells (Fig. 1A). B16 cells were not sensitive to C-CPE-PSIF (Fig. 2A). We transfected claudin-4 cDNA into B16 cells and established stable CL4-B16 cells (Fig. 1A). The proliferation rate was not affected by exogenously expressed claudin-4 (Fig. 1B). Metastasis has multiple processes, including motility and invasion (Steeg, 2006). To investigate the invasion of CL4-B16 cells, we performed a Boyden chamber migration assay. Cells were seeded onto the cell culture insert of Matrigel-coated membrane with an 8-μm pore size, and the cells that invaded the apical membrane and reached the basal membrane were counted. As shown in Fig. 1C, the invasion activity was increased 17-fold in CL4-B16 cells compared with that in parental B16 cells. Lung metastasis of CL4-B16 cells was observed when intravenously injected into mice; however, the number of lung metastasis colonies of CL4-B16 cells was smaller than that of the parental B16 cells (Fig. 1D). These findings indicate that CL4-B16 cells can be used as a metastasis model of claudin-4-expressing cancer cells. We discuss the elevation of migration activity and lower lung metastasis in CL4-B16 cells under Discussion.

Antitumor Activity of C-CPE-PSIF in CL4-B16 Cells. Before in vivo experiments, we investigated the in vitro cytotoxicity of C-CPE-PSIF in CL4-B16 cells. As shown in Fig. 2A, C-CPE-PSIF showed dose-dependent cytotoxicity in CL4-B16 cells, decreasing their viability to 35% at 100 ng/ml. In contrast, parental B16 cells were not sensitive to C-CPE-PSIF even at 1 μg/ml, indicating that C-CPE-PSIF may target claudin-4. Claudin-4 is expressed in the intestines, liver, and kidney (Morita et al., 1999). To determine a safe dose of C-CPE-PSIF, we checked serum biochemical markers of liver (alanine aminotransferase) and kidney (blood urea nitrogen) injury in mice injected with C-CPE-PSIF. After intravenous administration of C-CPE-PSIF (5 μg/kg), the mice showed no signs of injury (data not shown). In the following in vivo experiments, the doses of C-CPE-PSIF were less than or equal to 5 μg/kg. B16 or CL4-B16 cells were intravenously injected into mice, and then C-CPE-PSIF was intravenously administered every 2 days. Two weeks after the tumor cell injection, the number of lung metastasis colonies was counted. As shown in Fig. 2B, C-CPE-PSIF treatment decreased the number of lung metastasis colonies from 39 ± 17 to 10 ± 4 at 5 μg/kg. In contrast, C-CPE-PSIF treatment did not affect the lung metastasis of B16 cells (Fig. 2B). C-CPE-PSIF suppressed the growth of CL4-B16 cells but not B16 cells inoculated into the right flank of mice (Fig. 2C). These data suggest that claudin-4 targeting may be a potent strategy for suppressing tumor metastasis and growth.

Suppression of the Primary Tumors and Metastasis of 4T1 Cells. To clarify the potency of a claudin-4-targeting strategy, we investigated the effect of C-CPE-PSIF on a spontaneous metastasis model. 4T1 cells are murine cancer cells that spontaneously metastasize to the lung after being subcutaneously inoculated (Wong et al., 2002). We investigated whether C-CPE-PSIF suppresses the spontaneous lung metastasis of 4T1 cells. On day 33, the tumor volume was 1801.2 ± 848.5 mm³ in the vehicle-treated group and 740.5 ± 94.6 mm³ in the group treated with 5 μg/kg C-CPE-PSIF (Fig. 3A). The number of lung metastasis colonies was decreased to 2 ± 1 colonies at 5 μg/kg C-CPE-PSIF (Fig. 3B). A dose of 2 μg/kg C-CPE-PSIF did not suppress tumor growth
but did prevent lung metastasis. The circulating tumor cells might be more sensitive to C-CPE-PSIF than tumor cells in the solid tumor tissue. C-CPE-PSIF treatments did not cause a decrease in body weight (Fig. 3C), and there were no apparent biochemical side effects (Supplemental Fig. 1). ADR, which is frequently used in clinical chemotherapy, suppressed the tumor growth from 970.3 ± 278.4 to 458.6 ± 51.4 mm³ at 4 mg/kg (Fig. 3D). As shown in Fig. 3E, 4 mg/kg ADR decreased the number of lung metastasis colonies (24 ± 13 colonies in the vehicle-treated group; 6 ± 4 colonies in the ADR-treated group). However, the ADR-treated mice experienced a 26% loss of body weight, which is a sign of side effects (Fig. 3F). Thus, the antitumor activity of C-CPE-PSIF might be more potent than that of ADR. These results indicate that claudin-4-targeting therapy might be a potent strategy for tumor therapy with a low level of side effects and a high level of antitumor activity.

Discussion

Most malignant tumors are derived from the epithelium, and metastasis is the major cause of death from cancers. In the present study, we found that systemic administration of a claudin-targeting molecule suppressed cancer metastasis, indicating that claudin targeting might be an effective therapy against cancer metastasis.
Although the in vitro metastasis activity of CL4-B16 cells was higher than that of parental B16 cells, the in vivo lung metastasis of CL4-B16 cells was lower than that of B16 cells. As shown in Supplemental Fig. 2, claudin-4 increased the invasiveness and migration activity of B16 cells in vitro and decreased lung metastasis in vivo. A possible explanation for this discrepancy might be the experimental model, which evaluates a different stage of metastasis. The migration and invasion activity involved in the early stage of metastasis was investigated in the in vitro analysis, whereas extravasation and colonization to an organ involved in the late stage of metastasis was evaluated by the in vivo experiment. The altered expression of claudin-4 changed the metastasis of 4T1 cells to the heart and liver, suggesting that claudin affects organ-specific metastasis (Erin et al., 2009). Claudin-4 might suppress the lung metastasis of B16 cells.

Claudin is a structural and functional component of TJs (Furuse and Tsukita, 2006). What is the role of claudin in metastasis? Metastasis is composed of three steps: leaving the primary site, entering the blood flow, and invading the distant site. In the first step, the combination of claudin members in the TJ strands may be important. The claudin family contains at least 24 members. Claudin is believed to form homo- and hetero-type claudin strands, and the pattern of the strands differs among tissues and determines the properties of TJ seals (Furuse and Tsukita, 2006). For example, rigid TJ seals were formed when claudin-11 or claudin-15 was exogenously expressed in Madin-Darby canine kidney cells, whereas the expression of claudin-11 or claudin-15 reduced the TJ integrity in LLC-PK1 cells by its dominant negative effects on TJ sealing (Van Itallie et al., 2003). Dominant negative effects of claudin-4 on the TJ barrier might contribute to an acceleration in the detachment of cancer cells from the primary tumor tissue. In the second and third steps of metastasis, cancer cells must move through the extracellular matrix at the primary site and the distant site. Cancer cells must degrade the extracellular matrix by the expression of matrix metalloproteinase and increase their motility. Claudin expression enhanced invasion with increased matrix metalloproteinase activity (Agarwal et al., 2005). There is a relationship between the levels of claudin-1/claudin-4 and the metastasis of human cancers, including hepatic, colonic, ovarian, and gastric cancers (Agarwal et al., 2005; Resnick et al., 2005; Halder et al., 2008; Lee et al., 2008). The overexpression of claudin suppressed cancer metastasis in human pancreatic and gastric cancers (Michl et al., 2003; Mima et al., 2005; Ohtani et al., 2009). Claudin-4 suppressed or accelerated in vitro and in vivo metastasis of
human cancer cells (Agarwal et al., 2005; Ohtani et al., 2009). Cell–cell interaction through TJs regulates cell growth signaling (Matter et al., 2005). Taken together, these findings indicate that claudin family members might control several steps of cancer metastasis. The precise molecular mechanism and role of claudin in cancer metastasis remain to be determined.

Whether a claudin-4-targeting method causes severe side effects is critical for its clinical application in cancer therapy. Claudins play pivotal roles in TJ barrier and fence functions by maintaining cellular polarity in normal epithelium (Furuse and Tsukita, 2006). Claudins are believed to be more accessible in tumors than in normal epithelium. Claudins form TJ seals in lateral membranes between adjacent cells in normal epithelium, whereas Claudins are exposed on the cell surface during tumorigenesis (Soler et al., 1999; Kominsky, 2006). Indeed, no local or systemic side effects have been observed after the intratumoral administration of CPE (Kominsky et al., 2007; Santin et al., 2007). Here, we also found that the systemic administration of C-PCE-PSIF causes no significant increase in biochemical markers (aspartate aminotransferase, alanine aminotransferase, and blood urea nitrogen) for toxicity at a therapeutic dose of 5 mg/kg (Supplemental Fig. 2). Thus, a claudin-targeting strategy might have weak side effects.

It is difficult to prepare recombinant claudin protein because of its hydrophobic property, and claudin has low antigenicity. Until recently, an antibody against the extracellular loop domain of claudin had never been successfully prepared, and C-PCE was the only known claudin binder (Sonoda et al., 1999). Recently, Romani et al. (2009) prepared a single-chain antibody fragment against claudin-3 by using phage display technology. They found that the antibody fragment binds to ovarian and uterine carcinoma cells in vitro. More importantly, a therapeutic monoclonal antibody against claudin-4 was developed. Suzuki et al. (2009) successfully prepared anti-claudin-4 antibody by immunizing claudin-4-expressing tumor cells into a mouse with autoimmune disease. The antibody mediates anadhesive and cell-cell cytotoxicity and both in vitro and in vivo antitumor activity. Although the preparation of anticlaudin antibody may lead to a breakthrough in cancer therapy, the immunogenicity associated with immunotoxin clinical therapies is a future problem (Kreitman and Pastan, 2006). The C-terminal 30 amino acids are the minimum functional domain of C-PCE to bind to claudin-4 (Hanna et al., 1991). The C-terminal 30-amin acid fragment was used to deliver a cytotoxin to claudin-4-expressing cells by genetic fusion (Yuan et al., 2009). Humanized antibody and the claudin-4-targeting peptide may be useful for cancer therapy in the near future. In summary, this is the first report to indicate that systemic injection of a claudin-targeting molecule suppresses tumor growth and metastasis. Hematologic cells do not develop TJs; therefore, a claudin-targeting therapy may have no hematologic toxicity. We anticipate that claudin targeting will be a potent strategy for cancer therapy.

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References


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Supplemental data

Title: A claudin-targeting molecule as an inhibitor of tumor metastasis
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Hidehiko Suzuki, Yohei Kakamu, Akihiro Watari, Kiyohito Yagi
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Supplemental Figure 1. Toxicity of C-CPE-PSIF in liver and kidney
Female BALB/c mice (7 to 8 weeks old) were intravenously injected with C-CPE-PSIF at the indicated dose once (A) or three times per week for 2 weeks. Seventy-two hours after the last injection, the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were measured by using a Test-WAKO kit. Data are presented as the mean ± SD (n=5). Data are representative of at least two independent experiments.
Supplemental Figure 2. Effects of claudin-4 on metastasis in B16 cells

A) Claudin-4-expressing B16 cells. CL4-B16 cell clones (clone 1 and 2) were lysed, and the cellular lysates were subject to SDS-PAGE, followed by western blotting with mouse-claudin-4 and β-actin. Clone 2 is identical to CL4-B16 in the Figures 1-3. B) Effect of claudin-4 expression on cell invasion. Cells (1 × 10^5 cells) were seeded into the upper well of the cell-culture insert coated with matrigel. After 24 h, the cells that invaded the bottom membrane of the insert were stained with DiffQuick reagent and counted under a microscope. Data are shown as means ± SD (n=4). *Significantly different from B16 cells (p < 0.01). C) Effect of claudin-4 expression on lung metastasis. Cells (5 × 10^5 or 1 × 10^6 cells) were injected into the tail veins of C57BL/6J mice. After 14 days, the mice were sacrificed, their lungs were fixed, and the colonies on the lung surface were counted. Data are shown as means ± S.D. (n=5). *Significantly different from B16 cells (p < 0.05).