enzymes in various tissues, including the artery, pancreatic islets, and brain [11–13]. A variety of types of gene delivery vehicles have been employed for delivery of antioxidative genes so far, and replication-incompetent adenovirus (Ad) vectors have several advantages over other vehicles to deliver antioxidant genes to the liver. First, Ad vectors have high tropism to livers. A more than 10^3-fold higher transgene expression is found in the liver, compared with other organs, following systemic administration [14–16]. Second, non-dividing cells are efficiently transduced with Ad vectors. Hepatocytes do not actively divide under normal conditions. Non-viral gene delivery vehicles have been used for prevention of hepatic I/R injury in previous studies [17,18]; however, non-viral gene delivery vehicles mediate inefficient transfection in non-dividing cells. Third, the Ad vector genome is not integrated into the host genome, indicating that transduction with Ad vectors is unlikely to induce insertional mutagenesis in hepatocytes. Fourth, Ad vector-mediated gene expression in liver persists for 1–2 weeks, [19,20] in contrast, rapid reduction in plasmid DNA-mediated transgene expression in organs is found after injection of non-viral gene delivery vehicles [21,22]. In spite of these advantages of Ad vectors, the ability of Ad vectors expressing antioxidant enzymes to prevent hepatic I/R injury has not been fully examined probably because Ad vectors are generally considered more toxic than non-viral gene delivery vehicles; however, our group demonstrated that intravenous administration of Ad vectors induces less amounts of inflammatory cytokines than cationic lipid/plasmid DNA complexes [23]. In addition, fiber-modified Ad vectors carrying a stretch of lysine residues in the C-terminus of a fiber knob have been demonstrated to poorly activate innate immune responses after systemic injection, compared with conventional Ad vectors [24]. These results suggest that Ad vectors, including fiber-modified Ad vectors, would be suitable for prevention of I/R injury by delivering antioxidant genes to livers.

Among antioxidant enzymes, SOD is often used for detoxifying ROS in previous studies [8,17,25,26]. SOD catabolizes superoxide anion to H_2O_2; however, H_2O_2 is converted to hydroxyl radicals, which are extremely reactive and more toxic than other ROS. H_2O_2 should be removed to effectively reduce I/R injury. Another antioxidant enzyme, catalase, prevents the generation of hydroxyl radicals by catabolizing H_2O_2 to H_2O and O_2, suggesting that catalase is promising for prevention of I/R injury. However, there are few studies reporting therapeutic effects of catalase gene delivery on I/R injury [17,27].

In the present study, catalase-expressing Ad vectors were intravenously pre-administered to prevent I/R-induced hepatic injury. Pre-injection of catalase-expressing Ad vectors successfully prevented not only I/R-induced hepatic injury but also CCl_4-induced liver damages. Furthermore, mice receiving pre-injection of catalase-expressing Ad vectors showed improved survival rates after partial hepatectomy followed by hepatic I/R.

2. Materials and methods

2.1. Cells

A549 (a human lung adenocarcinoma epithelial cell line), HepG2 (a human hepatocellular liver carcinoma cell line), and 293 (a human embryonic kidney cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum under 5% CO_2 at 37 °C.

2.2. Ad vectors

Ad vectors were constructed by means of an improved *in vitro* ligation method [28–30]. Briefly, the LacZ gene, which is derived from pCMVβ (Marker Gene, Inc., Eugene, OR) and the catalase gene, which is derived from pZEOSV2-CAT (a kind gift from Dr. J. Andres Melendez, Albany Medical College, Albany, NY) [31,32] were inserted into pHMCA5, [33] creating pHMCA5-LacZ and pHMCA5-CAT, respectively. pHMCA5-LacZ and pHMCA5-CAT were then digested with I-CeuI and PI-SceI, and ligated with I-CeuI/PI-SecI-digested pAdHM4 [28], resulting in pAdHM4-LacZ and pAdHM4-CAT, respectively. To generate the viruses, PacI-digested Ad vector plasmids were transfected into 293 cells plated in a 60-mm dish with SuperFect (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The viruses were prepared by the standard method, then purified with $CsCl_2$ gradient centrifugation, dialyzed with a solution containing 10 mM Tris (pH7.5), 1 mM $MgCl_2$, and 10% glycerol, and stored in aliquots at −80 °C. The determinations of infectious titers and virus particle (VP) titers were accomplished using 293 cells and an Adeno-X rapid titer kit (Clontech, Mountain View, CA) and the method of Maizel et al. [34], respectively. Catalase-, or β-galactosidase-expressing fiber-modified Ad vectors carrying a stretch of lysine residues (K7 (KKKKKKK) peptide) in the C-terminus of a fiber knob, AdK7-CAT and AdK7-LacZ, respectively, were similarly prepared using pAdHM41K7 [35]. The ratios of the biological-to-particle titer were 1:20, 1:31, 1:45, and 1:39 for Ad-LacZ, AdK7-LacZ, Ad-CAT, and AdK7-CAT, respectively.

2.3. Western blot analysis for catalase expression

A549 cells were transduced with Ad vectors at 3000 VP/cell for 2 h. Forty-eight hours later, cells were harvested and lysed with lysis buffer (20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 10% glycerol) containing protease inhibitor cocktail (Sigma Chemical., St. Louis, MO). Equal quantities of protein (5 μg), as determined by a protein assay (Bio-Rad, Hercules, CA), were subjected to sodium dodecyl sulfate/12.5% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking nonspecific binding, the membrane was incubated with anti-catalase antibody (diluted 1/8000; Calbiochem, San Diego, CA) at room temperature for 3 h, followed by reaction with horse radish peroxidase (HRP)-conjugated anti-rabbit IgG (diluted 1/3000; Cell Signaling Technology, Beverly, MA) at room temperature for 1 h. The band was visualized by ECL Plus Western blotting detection reagents (Amersham Bioscience, Piscataway, NJ), and the signals were read using an LAS-3000 imaging system (Fujifilm, Tokyo, Japan). For detection of the internal control, a polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase antibody (diluted 1/5000; Trevigen, Gaithersburg, MD) and an HRP-conjugated anti-rabbit IgG were used.

2.4. In vitro protective effect of catalase-expressing Ad vectors on ROS-induced cell damage

HepG2 cells (5000 cells/well) were seeded onto a 96-well plate. On the following day, the cells were transduced with Ad-LacZ, AdK7-LacZ, Ad-CAT, or AdK7-CAT at 300 or 3000 VP/cell for 2 h. After a 48-h incubation, the medium was exchanged for normal medium containing 30 mM menadione (Sigma Chemical), which is a ROS inducer. On the following day, the cell viability was determined by Alamar blue staining (BioSource, San Diego, CA).

2.5. Catalase activities in the liver after intravenous administration of Ad vectors

Ad vectors (Ad-LacZ, AdK7-LacZ, Ad-CAT, and AdK7-CAT) were intravenously administered into C57BL/6 mice (7–8-week-old females; Nippon SLC, Shizuoka, Japan) at a dose of 1×10^{10} VP/mice. Forty-eight hours later, the livers were isolated and homogenized with 50 mM potassium phosphate buffer containing 1 mM EDTA. The supernatants were recovered after centrifugation of the homogenates, and catalase activity in the supernatants was measured using a CalBiochem Catalase Assay Kit (Calbiochem).

2.6. Hepatic ischemia/reperfusion experiment

Mice were intravenously administered PBS (control) or Ad vectors via the tail vein at a dose of 10^{10} VP/mice. A partial hepatic ischemia/reperfusion experiment was performed as previously described [36,37]. Briefly, 2 days post-administration of Ad vectors, mice were anesthetized with a peritoneal injection of pentobarbital sodium (50 mg/kg). An incision was made in the abdomen, and all structures in the portal triad (hepatic artery, portal vein, bile duct) were occluded with a vascular clamp for 1 h to induce hepatic ischemia. Then, blood was allowed to flow through the liver again by removal of the clamp (reperfusion). After an appropriate period of reperfusion (0, 1, 6, 24 h), blood was collected via retro-orbital bleeding, and serum was obtained by centrifugation. The aspartate aminotransferase (ALT) and alanine aminotransferase (AST) activities in serum, as indicators of liver injury during reperfusion, were assayed using a transaminase-CII test (Wako, Osaka, Japan). In separate experiments, histology in the liver sections was evaluated 24 h after reperfusion. The livers were recovered and fixed by immersion in 10% buffered formalin, embedded in paraffin and processed for histology. Tissue damage was assessed in hematoxylin and eosin-stained sections. A sham surgery was performed under anesthesia but without occluding the vessels.

2.7. CCl_4-induced liver injury experiment

Ad vectors were intravenously administered into mice as described above. Forty-eight hours after Ad vector injection, CCl_4 dissolved in olive oil was intraperitoneally administrated to the mice at a dose of 1 ml/kg body weight to induce acute liver failure. Twenty-four hours after CCl_4 administration, blood was collected via retro-orbital bleeding, and the levels of ALT and AST in the serum were determined as described above.

2.8. Partial hepatectomy

Ad vectors were intravenously administered into mice as described above. Forty-eight hours after Ad vector injection, mice were anesthetized and subjected to two-thirds hepatectomy as described previously [38,39]. Subsequently, liver I/R was conducted by occlusion of the blood vessel to block the blood flow into the remnant liver for 8 min followed by reperfusion as described above. After the surgery, the mice were maintained under conventional conditions to monitor survival rates.

2.9. Statistical analysis

Results were expressed as the means ± S.D. Statistically significant differences between groups were determined by the two-way analysis of variance, followed by Student's *t*-test. The levels of statistical significance were set at $p<0.05$ and $p<0.01$.

3. Results

3.1. Ad vector-mediated catalase expression in vitro

First, to examine *in vitro* catalase expression levels following Ad vector infection, Western blotting analysis was performed. We observed an apparent increase in the catalase expression after transduction with Ad-CAT or AdK7-CAT in A549 cells (Fig. 1). In addition, AdK7-CAT mediated higher catalase expression than Ad-CAT, probably due to the higher transduction activity of AdK7 vectors than conventional Ad vectors [35]. The control Ad vectors, Ad-LacZ and AdK7-LacZ, did not increase catalase expression, indicating that transduction with Ad vectors alone does not induce any change in the antioxidant systems.

Next, to examine whether Ad vector-mediated over-expression of catalase prevents ROS-induced cellular toxicity, the cells were incubated with 30 mM menadione following transduction with Ad vectors, and the cell viabilities were determined. It is well known that menadione produces superoxide radicals in cells, leading to oxidative stress-induced cell death [40,41]. As shown in Fig. 2, the cell viability was significantly reduced to less than 50% in the presence of 30 mM menadione. In contrast, transduction with catalase-expressing Ad vectors dramatically improved the cell viabilities. Transduction with Ad-CAT and AdK7-CAT at 300 VP/cell resulted in cell viabilities of 70.8% and 79.1% of the cell, respectively. These results indicate that Ad vector-mediated over-expression of catalase is beneficial in preventing oxidative stress-induced cell death by efficiently deleting ROS.

3.2. Catalase activity in the liver following catalase-expressing Ad vector injection

Next, to measure catalase activities in the liver following intravenous administration of Ad vectors, the livers were recovered 48 h after Ad vector injection, and catalase activities in the liver were determined. The catalase activities were 2.4-fold and 4.3-fold increased following administration of Ad-CAT and AdK7-CAT, respectively (Fig. 3). By contrast, we found no elevation in the catalase activity by LacZ-expressing Ad vectors. These results indicate that catalase activity in the liver is significantly elevated by intravenous administration of catalase-expressing Ad vectors.

3.3. Prevention of hepatic I/R injury by pre-administration of catalase-expressing Ad vectors

To evaluate the ability of catalase-expressing Ad vectors to prevent hepatic I/R injury, serum ALT and AST levels were measured after 1 h of hepatic ischemia followed by reperfusion. Both serum ALT and AST levels were highly elevated at 1 h and 6 h after the reperfusion of hepatic flows in the mice pre-injected with PBS, indicating that hepatic injury was induced by I/R (Fig. 4). At 1 h after reperfusion, the ALT and AST levels increased from 59.3 to 184.0 and from 421.2 to 1174.7 IU/L, respectively. However, pretreatment with Ad-CAT or AdK7-CAT significantly reduced the serum ALT and AST levels at 6 h after reperfusion. The ALT and AST levels in mice pre-injected with AdK7-CAT were 3.9- and 4.4-fold lower than those in mice pre-injected with PBS. Reductions in the ALT and AST levels were also observed at 1 h after reperfusion, although these changes were not statistically significant. The control Ad vectors, Ad-LacZ and AdK7-LacZ, exhibited no suppressive effects on the I/R-induced elevation of serum ALT and AST levels.

Furthermore, to histologically evaluate the preventive effects of catalase-expressing Ad vectors, liver sections were prepared 24 h after reperfusion. An extensive necrotic area was observed in the mice pretreated with PBS or AdK7-LacZ (Fig. 5B, C). In contrast, transduction with Ad-CAT or AdK7-CAT resulted in a dramatic decrease in the necrotic area induced by hepatic I/R (Fig. 5D, E). In particular,

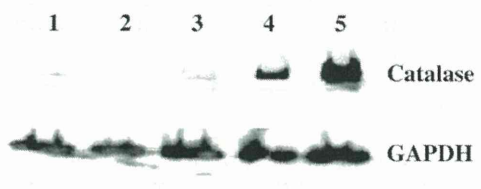

Fig. 1. Catalase expression following Ad vector transduction. A549 cells were transduced with Ad-LacZ, Ad-CAT, or AdK7-CAT at 3000 VP/cell for 2 h. Protein samples were collected after a 48-h incubation and analyzed by Western blotting. Lane 1, mock; lane 2, Ad-LacZ; lane 3, AdK7-LacZ; lane 4, Ad-CAT; lane 5, AdK7-CAT. The results are representative of two

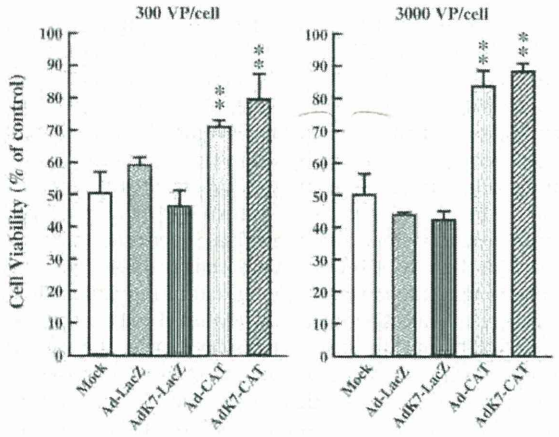

Fig. 2. Protective effects of catalase-expressing Ad vectors against menadione-induced cell death. HepG2 cells were transduced with Ad vectors at 300 or 3000 VP/cell for 2 h. After a 48-h incubation, menadione was added to the medium at a final concentration of 30 mM, and the cells were cultured for an additional 24 h. The cellular viabilities were then determined by Alamar blue staining. The cellular viabilities were normalized to the viability of Ad vector-infected HepG2 cells in the absence of menadione. The data are expressed as the means ± S.D. ($n=4$). **Significantly different from the mock-infected group at $p<0.01$.

pre-injection of AdK7-CAT almost completely prevented necrosis in the liver, although there were several small necrotic areas in the liver pretreated with Ad-CAT, probably due to the higher transduction efficiency and less liver toxicity profile of AdK7 vectors in the liver compared with conventional Ad vectors [24]. A TUNEL assay indicated that Ad-CAT and AdK7-CAT prevented the DNA fragmentation caused by hepatic I/R in hepatocytes (data not shown). These results indicate that the I/R-induced histological damages were also significantly attenuated by pretreatment with catalase-expressing Ad vectors.

3.4. Preventive effect of catalase-expressing Ad vectors on CCl_4-induced liver injury

To explore whether Ad vector-mediated catalase expression prevents other types of oxidative stress-induced liver injury, CCl_4 was intraperitoneally injected into mice pretreated with catalase-expressing Ad vectors. CCl_4 is well known to produce CCl_3 radical, leading to acute liver injury. Serum ALT and AST levels were highly elevated following CCl_4 treatment in mice pretreated with PBS or LacZ-expressing Ad vectors (Fig. 6). However, serum ALT and AST levels were markedly reduced by pretreatment with Ad-CAT and AdK7-CAT. AdK7-CAT mediated a 4.9-fold and 3.9-fold reduction in serum ALT and AST levels, respectively, compared with PBS. Ad-CAT and AdK7-CAT also mediated a dramatic improvement of CCl_4-induced gross abnormality in the liver (data not shown). These results indicate that catalase-expressing Ad vectors possess preventive effects on oxidative stress-induced injury that are distinct from their effects on I/R injury.

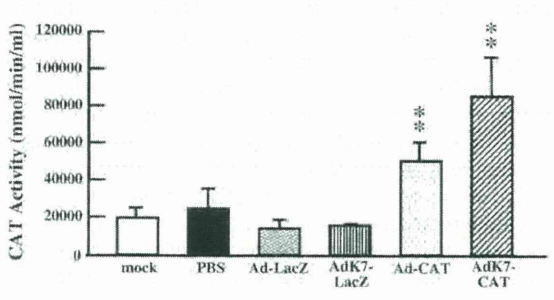

Fig. 3. Catalase activity in the liver following intravenous administration of catalase-expressing Ad vectors. Ad vectors were administered to mice at the dose of 1×10^{10} VP/mouse. The livers were recovered 48 h after injection, and catalase activities in mouse liver homogenates were determined. The data are expressed as the means ± S.D. ($n=5$). **Significantly different from the mock-infected group at $p<0.01$.

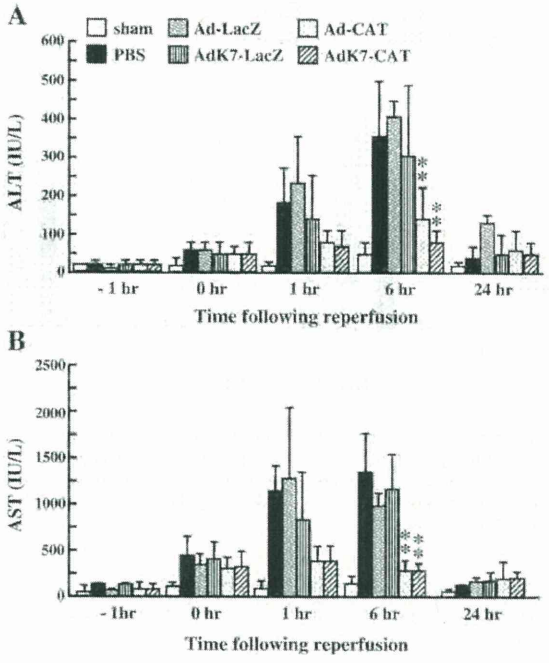

Fig. 4. Effects of pre-administration of catalase-expressing Ad vectors on serum ALT (A) and AST (B) levels in mice following hepatic ischemia/reperfusion injury. Ad vectors were intravenously administered to mice at a dose of 1×10^{10} VP/mice. Forty-eight hours after Ad vector injection, mice were subjected to a 1 h period of ischemia followed by hepatic reperfusion. Serum samples were taken at 1 h before ischemia, and 0, 1, 6, and 24 h after reperfusion. The data are expressed as the mean ± S.E. ($n=3$–8). **Significantly different from the PBS-injected group at $p<0.01$.

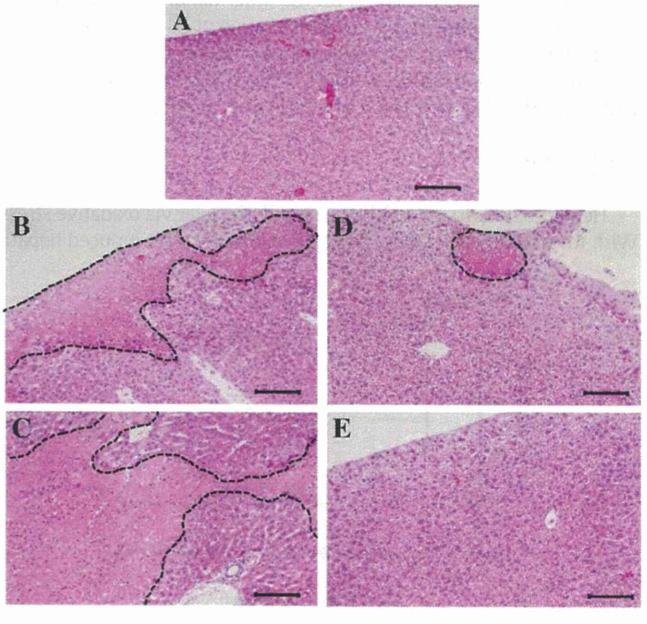

Fig. 5. Representative images of liver sections of mice 24 h following hepatic ischemia/reperfusion. A) Sham, B) PBS, C) AdK7-LacZ, D) Ad-CAT, and E) AdK7-CAT. Mice were subjected to hepatic I/R 48 h after Ad vector injection, as described in Fig. 4. Livers were recovered 24 h after I/R treatment, and liver sections stained with hematoxylin and eosin were observed under a microscope. A dashed line indicates the necrotic area. The scale bar represents 100 μm.

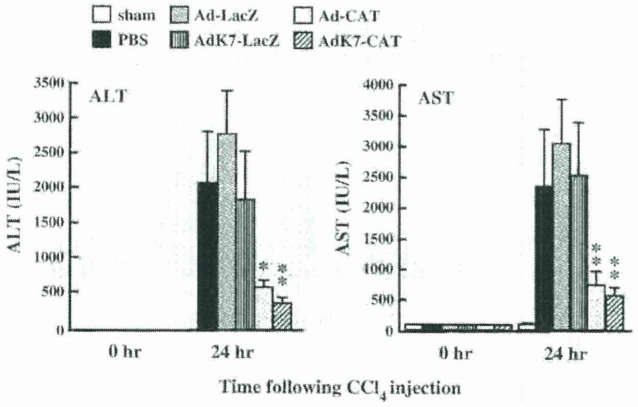

Fig. 6. Preventive effect of catalase-expressing Ad vectors on CCl_4-induced acute liver failure. CCl_4 (1 ml/kg) was intraperitoneally injected to mice 48 h following Ad vector injection. Serum samples were collected 24 h after CCl_4 administration. The data are expressed as the means ± S.D. ($n=4$). *Significantly different from the PBS-injected group at $p<0.05$; ** at $p<0.01$.

3.5. *Improvement of survival rates of mice subjected to partial hepatectomy and hepatic ischemia/reperfusion by catalase-expressing Ad vectors*

To examine whether over-expression of catalase improves the remnant liver function in mice subjected to both partial hepatectomy and I/R treatment, partial hepatectomy and subsequent I/R treatment were conducted 48 h after pre-administration of Ad vectors. Partial hepatectomy is often performed under hepatic ischemia, and the remaining liver suffers from I/R injury after partial hepatectomy in clinical settings. Mice pre-administered with AdK7-CAT showed a dramatic improvement in survival rate (Fig. 7). Seventy percent of mice survived for 7 days after these treatments. The body weights of the mice pre-injected with AdK7-CAT were not significantly reduced 7 days after surgery, compared with those before surgery (data not shown), suggesting that the general health of the mice was not substantially compromised after the surgery. On the other hand, the survival of the mice was not prolonged by pre-administration of Ad-LacZ or PBS. These results indicate that catalase-expressing Ad vectors are able to protect the liver from more serious stress induced by partial hepatectomy and I/R, and to improve the remnant liver function.

4. Discussion

Hepatotoxins, drugs, and I/R can injure the liver via oxidative stress. With the aim of efficiently preventing oxidative stress-induced hepatic injury, we pre-administered Ad vectors expressing an antioxidative enzyme, catalase, to mice. The results of our study demonstrated that Ad vector-mediated over-expression of catalase in the liver effectively prevents hepatic injury caused by not only I/R but also CCl_4. Furthermore, the survival rates of mice subjected to both partial hepatectomy and I/R treatment were prolonged by over-expression of catalase.

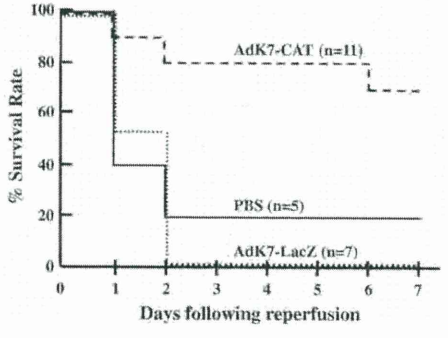

Fig. 7. Survival rates of mice subjected to partial hepatectomy and hepatic I/R following Ad vector administration. Solid line: PBS ($n=5$); dotted line: AdK7-LacZ ($n=7$); dashed line: AdK7-CAT ($n=11$). Ad vectors were intravenously administered to mice as described in Fig. 4. Forty-eight hours after Ad vector administration, mice were subjected to two-thirds partial hepatectomy, followed by an 8 min-period of ischemia

Superoxide anion is the primary oxidant species generated during hepatic I/R by a xanthine oxidase system and/or decoupling of the electron transport system in mitochondria. Superoxide anion is readily converted to H_2O_2 by SOD or a spontaneous reaction. H_2O_2 itself is a weak oxidizing agent; however, hydroxyl radical is produced by H_2O_2 in the presence of transition-metal ion. Hydroxyl radical has the most oxidative ability and the strongest toxicity among the various ROS. Reduction/elimination of hydroxyl radical is considered to be the most effective strategy for prevention of hepatic I/R injury. Therefore, catalase, which prevents generation of hydroxyl radical by converting H_2O_2 to H_2O and O_2, was selected as the antioxidant enzyme in the present study. Catalase derivatives also exhibited higher preventive effects on the elevation of serum ALT and AST levels induced by hepatic I/R, compared with SOD derivatives [5,6]. In addition, over-expression of catalase in the liver might increase endogenous expression of SOD, which is another advantage of catalase gene transfer. He et al. demonstrated that delivery of catalase gene alone to the liver induced SOD activity in the liver [17].

Partial hepatectomy is often performed under hepatic ischemia. Previous studies have shown that oxidative stress induced by hepatic I/R affects hepatocyte cell death and inhibits liver regeneration [42,43]. Beyer et al. reported that ROS are directly responsible for the impairment of insulin/insulin-like growth factor 1 signaling, which is crucial for liver regeneration [44]. Furthermore, hepatocytes without catalase activity have been found in regenerating livers after partial hepatectomy [45], suggesting that hepatocytes in the regenerating livers might be susceptible for ROS-mediated injury. The present study demonstrated that over-expression of catalase in the liver dramatically improved the survival rates of mice subjected to partial hepatectomy and I/R, suggesting that the remnant livers would be protected from ROS-mediated injury by over-expression of catalase. Furthermore, over-expression of catalase might play an important role in maintenance of the regenerative capacity of hepatocytes. Recently, removal of ROS by antioxidant enzymes was demonstrated to be crucial for maintenance of the self-renewal capacity of progenitor/stem cells in an *in vitro* culture system [46,47]. In this study, the liver/body weight ratio of mice pre-injected with AdK7-CAT 1 week after partial hepatectomy and I/R was 4.7 ± 0.55%, which is not significantly different from that of naïve mice (data not shown).

Oxidative stress is also generated in the liver through metabolism of a variety of drugs, chemicals, and toxins, such as thioacetamide, lipopolysaccharide, and CCl_4. In particular, CCl_4 is often used as a representative hepatotoxin causing oxidative stress in animal experiments. CCl_4 is metabolized by cytochrome P450 in the endoplasmic reticulum of hepatocytes, leading to generation of CCl_3 radical, which induces hepatic damages, although the mechanism of CCl_4-mediated liver damage has not yet been fully revealed. The present study showed that Ad vector-mediated over-expression of catalase in the liver also attenuated CCl_4-induced liver injury (Fig. 6). Over-expression of SOD has also been shown to inhibit CCl_4-induced hepatic damages [48]. Hepatic delivery of genes encoding ROS-deleting enzymes is effective in case of hepatic injury induced by oxidative stress-generating hepatotoxins and chemical compounds.

Ad vectors offer various advantages for gene delivery to the liver; however, systemic administration of Ad vectors often induces inflammatory cytokine production and hepatic damage [24,49–51]. However, we found no apparent Ad vector-induced damages in the liver in this study (data not shown). Moreover, mice pre-administered Ad-LacZ or AdK7-LacZ did not exhibit higher levels of serum ALT and AST after I/R than those pre-administered PBS (Fig. 4). This was likely

due to the relatively low dose of Ad vectors (1×10^{10} VP/mouse) used in this study. Higher doses of Ad vectors have often been administered in the studies reporting Ad vector-mediated *in vivo* toxicities [49,51]. We confirmed that more than 90% of hepatocytes were efficiently transduced even at a dose of 1×10^{10} VP/mouse in this study (data not shown). In general, Ad vectors have been considered more toxic than non-viral vectors containing plasmid DNA; however, our group recently revealed that Ad vectors induced smaller amounts of inflammatory cytokines, which are partly involved in Ad vector-induced hepatic toxicity, following intravenous administration into mice, compared with plasmid DNA/cationic liposome complexes, which were used as a representative of non-viral gene delivery vehicle [23]. In both that previous study and our present work, Ad vectors successfully deliver antioxidant genes to the liver with no apparent toxicity, although we should pay attention to the vector doses.

In addition to conventional Ad vectors, fiber-modified AdK7 vectors, which display a poly-lysine motif on the c-terminal of the fiber knob, [24,35,52] were used in this study. AdK7 vectors mediate not only significantly higher transgene expression in the liver but also lower *in vivo* damages than conventional Ad vectors [24]. However, the serum ALT and AST levels of the mice receiving Ad-CAT and AdK7-CAT were not significantly different from those of the controls in this study (Fig. 4), although histological analysis of the liver sections revealed that AdK7-CAT conferred superior protection against I/R injury (Fig. 5), probably because sufficient levels of catalase would be expressed by Ad-CAT at this dose to prevent hepatic I/R-induced increases in serum ALT and AST in this setting. AdK7-CAT might show higher protective effects than conventional Ad vectors under more severe conditions.

In conclusion, the present study demonstrated that Ad vector-mediated catalase expression in the liver significantly improved I/R-induced hepatic injury. These findings suggests that Ad vectors expressing antioxidant enzymes would contribute to the development of strategies aimed at inhibiting hepatic I/R injury. Antioxidant enzyme-expressing Ad vectors are also applicable for the prevention of I/R-induced injury in other organs and oxidative stress-induced damages caused by ROS-generating chemicals. Moreover, their combined use with other antioxidant genes or anti-apoptosis genes could further attenuate oxidative stress-induced injury.

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Potency of Claudin-targeting as Antitumor Therapy

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Abstract

Approximately 90% of malignant tumors are derived from the epithelium. Epithelium has well-developed tight junctions (TJs), which become deregulated and disrupted during epithelial transformation. Claudin-4, a pivotal functional and structural component of TJs, is often overexpressed in human cancers. In the present study, we prepared a claudin-4 binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), fused to a cytotoxic molecule (C-CPE-PSIF) and found that C-CPE-PSIF is toxic to claudin-4-expressing cells. Interestingly, C-CPE-PSIF was less toxic in polarized than depolarized cells, and treatment of polarized cells with C-CPE-PSIF from the basal but not apical side was cytotoxic. C-CPE-PSIF also exhibits antitumor activity. C-CPE mutants with alanine substitutions were more cytotoxic than C-CPE. These findings indicate that the claudin-4 binder, C-CPE, is a potent lead molecule for the development of claudin-targeted tumor therapy.

Keywords: Claudin; *Clostridium perfringens* enterotoxin; *Pseudomonas* exotoxin; Anti-tumor activity

Introduction

Tight junctions (TJs) consist of three main classes of proteins: claudins, occludin, and junctional adhesion molecules. Claudins regulate the integrity and function of TJs (1). The human family of claudin proteins contains at least 23 members, and their expression profiles and functions are tissue and cell specific (2). Changes in claudin expression occur in malignant tumors, and the expression profiles differ among tissues (3, 4). For example, claudin-1 and -10 are overexpressed in colon and hepatocellular cancer cells, respectively (3), while claudin-4 is up-regulated in breast, ovarian, pancreatic, colorectal, hepatic and bladder cancers (Table 1). Thus, claudins are potential targets for tumor therapy. However, claudin has low antigenicity, and it is difficult to generate antibodies to the extracellular region of claudins.

Clostridium perfringens enterotoxin (CPE) is a single polypeptide of 35 kDa that causes food poisoning in humans. The functional domains of CPE are classified into the N-terminal cytotoxic and C-terminal receptor-binding regions (5, 6). Two years after the CPE receptor was identified in 1997, it was found to be identical to claudin-4 (7, 8). Interestingly, C-terminal CPE, which corresponds to amino acids 184 to 319 ($C\text{-}CPE_{184\text{-}319}$), binds to claudin-4 and inhibits TJ function. We previously found that $C\text{-}CPE_{184\text{-}319}$ is a useful ligand for claudin-4 (9). However, $C\text{-}CPE_{184\text{-}319}$ has poor solubility (0.3 mg/ml) and is difficult to use in pharmaceutical therapy. In 2008, Van Itallie et al. showed that a slightly smaller polypeptide, $C\text{-}CPE_{194\text{-}319}$, has high solubility (>10 mg/ml) and high affinity to claudin-4 (10).

Pseudomonas aeruginosa exotoxin A (PE) is widely used in cancer-targeting study (11). PE binds to the cell surface and is internalized via endocytosis; then, a PE fragment, protein synthesis inhibitory factor (PSIF), escapes from the endosome to the cytosol (12), where it inhibits protein synthesis by inhibiting elongation factor 2. PSIF lacks the receptor-binding domain of PE, and fusion of a tumor

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Table 1 Changes in expression of claudin-4 in cancer cells

Cancer	Expression	References
Prostate	Up	(25)
Breast	Up	(26, 27)
Ovarian	Up	(28-31)
Pancreatic	Up	(32-34)
Colorectal	Up	(35)
Uterus	UP	(36, 37)
Gastric	Down	(38, 39)
Hepatocellular	Up	(40)

antigen ligand with PSIF is a promising strategy for cancer-targeting therapy.

In the present study, we genetically prepared a claudin-4-targeting molecule (C-CPE$_{194-319}$-PSIF) containing the claudin-4-binding region of CPE and PSIF (Figure 1A). We also investigated whether C-CPE$_{194-319}$ (referred to as C-CPE hereafter) is useful for claudin-4-targeted cancer therapy.

Results and Discussion

L cells, which are a mouse fibroblast cell line, do not express any claudins. Therefore, we investigated the cytotoxicity of C-CPE-PSIF in claudin-4-expressing L cells (CL4/L cells). C-CPE-PSIF caused dose-dependent cytotoxicity in CL4/L cells, reaching >90% cell death at 10 ng/ml. In contrast, even at 20 ng/ml, PSIF was not cytotoxic to CL4/L cells (13). C-CPE-PSIF showed specific toxicity in CL4/L cells, and pretreatment of the cells with C-CPE attenuated the C-CPE-PSIF-induced cytotoxicity, indicating that C-CPE-PSIF may interact with claudin-4 via its C-CPE domain (13).

Claudin-4 is expressed in tissues including the lung, intestine, liver, and kidney. Most claudins in normal cells are contained in TJ complexes, whereas the localization of claudin is deregulated in some cancers. C-CPE-PSIF may recognize the deregulated localization of claudin-4. Confluent Caco-2 cells form a polarized cell monolayer with well-developed TJs, and they are frequently used as a model of polarized cells. Confluent Caco-2 cells express more claudin-4 than pre-confluent cells; however, C-CPE-PSIF was more toxic in the pre-confluent cells with fewer TJs (47% cell death at 5 ng/ml) than in the confluent cells with well-developed TJs (40% cell death, even at 200 ng/ml) (13).

Early events in epithelial carcinogenesis are deregulation of cellular polarity and loss of TJ structures. We examined whether the sensitivity of cells to C-CPE-PSIF is affected by their cellular polarity using 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. After the addition of C-CPE-PSIF to the apical or basolateral compartment of the Transwell chamber, we assessed the TJ barrier function of the cell sheets by measuring transepithelial electric resistance (TER). When C-CPE-PSIF was added to the apical compartment, TER was not affected for 48 h. In contrast, the addition of C-CPE-PSIF to the basolateral compartment caused a significant and dose-dependent reduction in TER. Furthermore, the addition of C-CPE-PSIF to the basolateral compartment, but not the apical compartment, increased the amount of released lactate dehydrogenase, a marker of cytotoxicity (13). These results indicate that C-CPE-PSIF has specific effects based on the cellular density and polarity.

We examined the antitumor activity of C-CPE-PSIF in 4T1 cells, a mouse breast cancer cell line that expresses claudin-4. To clarify the antitumor activity of the claudin-4-targeting molecule (C-CPE-PSIF), we prepared a fusion protein of PSIF with mutant C-CPE, in which Tyr306 and Leu315 (critical residues for the interaction between C-CPE

and claudin-4) were changed to alanines, resulting in C-CPE$_{Y306A/L315A}$-PSIF (14). C-CPE-PSIF mediated dose-dependent cytotoxicity in 4T1 cells, reaching 63% cell death at 100 ng/ml. In contrast, C-CPE$_{Y306A/L315A}$-PSIF was not cytotoxic 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. To investigate the in vivo antitumor activity of C-CPE-PSIF, 4T1 cells (2 x 10^6 cells) were inoculated into the right flank of mice on day 0. Vehicle, C-CPE, C-CPE-PSIF or C-CPE$_{Y306A/L315A}$-PSIF at a dose of 5 μg/kg was intratumorally injected on days 2, 4, 7, 9, 11, and 14. C-CPE-PSIF significantly suppressed tumor growth, and the tumor volume in the C-CPE-PSIF-treated group was 36% of that in the vehicle-treated group on day 16. In contrast, C-CPE and C-CPE$_{Y306A/L315A}$-PSIF, which lacked claudin-4-binding activity, had no effect on tumor growth, indicating that the antitumor activity of C-CPE-PSIF may depend on claudin-4 targeting (13).

We previously investigated the functional domains of C-CPE and found that the 16 C-terminal amino acids of C-CPE are involved in claudin-4 binding; we also found that the claudin-4 affinities of the N309A mutant, which contains Ala instead of Asn at position 309, and the S313A mutant, which contains Ala instead of Ser at position 313, were greater than that of C-CPE (14, 15). To improve the claudin-4-targeting molecule, we prepared C-CPE$_{S313A}$-PSIF and C-CPE$_{N309A/S313A}$-PSIF (Figure 1A). C-CPE$_{S313A}$-PSIF and C-CPE$_{N309A/S313A}$-PSIF were more cytotoxic than C-CPE-PSIF in CL4/L cells (Figure 1B). We are currently investigating the antitumor activity of these mutants and developing other C-CPEmutant-PSIF constructs with greater cytotoxicity. To develop novel methods of tumor diagnosis and therapies that target the initial stage of malignant transformation, we are also developing novel claudins binders, in addition to optimizing C-CPE, the conventional claudin-binder.

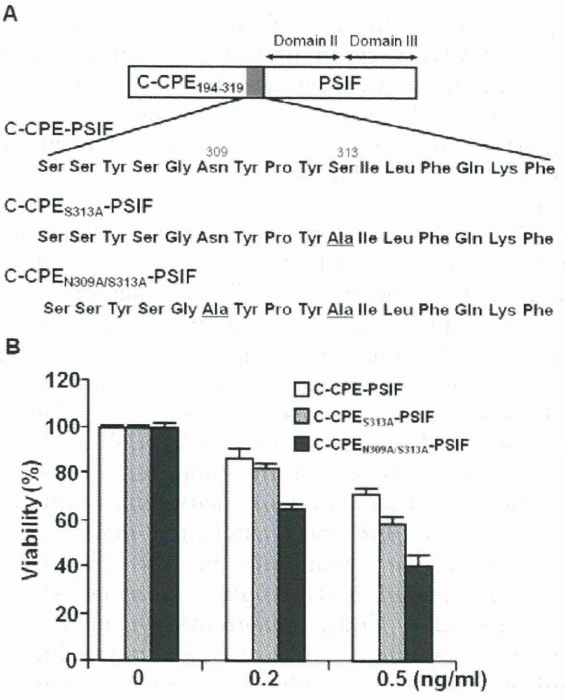

Figure 1. Cytotoxicity of C-CPE-PSIF fusion proteins (A) Schematic illustration of C-CPE-PSIF fusion proteins. C-CPE is the C-terminal fragment of CPE corresponding to amino acids 194-319. The dark area indicates the putative claudin-4-binding region (15). Changing Asn to Ala at position of 309 or Ser to Ala at position of 313 improved the affinity of C-CPE to claudin-4 (14). C-CPE$_{S313A}$ or C-CPE$_{N309A/S313A}$-fused PSIF was also prepared. PSIF contains domain II (the critical domain for the escape of the toxin from the endosome to the cytosol) and domain III (the critical domain for the inhibition of protein synthesis) of *Pseudomonas* exotoxin. (B) Cytotoxicity of C-CPE-PSIF. CL4/L cells were treated with the C-CPE-PSIF fusion proteins at the indicated concentration for 24 h. The cellular viability was measured by a WST-8 assay kit, according to the manufacturer's instructions (Nacalai Tesque, Kyoto, Japan). Viability (%) was calculated as a percentage of the vehicle-treated cells. The data represent the mean ± SD of three independent experiments.

Perspective of claudin-targeted tumor therapy

Approximately 7.6 million people worldwide die from cancer each year, and 90% of malignant tumors are derived from epithelial tissues (16) (http://www.reuters.com/article/healthNews/idUSN1633064920071217).

Cancer cells grow slowly during the very early stage of malignant transformation, after which they grow exponentially and form tumor tissues. Tumors that reach a mass of 10^{12} cancer cells lead to death. Diagnosable cancer tumors contain at least 10^9 cells, and tumors with 10^9-10^{12} cells are subject to cancer therapy. An important goal in cancer therapy is improving the ability to detect tumors containing less than 10^9 cells so that they can be more successfully treated.

Epithelial tissues are characterized by specific cellular polarity. The establishment and maintenance of cell polarity involve many processes, including signaling cascades, membrane trafficking events and cytoskeletal dynamics, and relies on the apical junctional system, TJs (17, 18). TJs seal the intercellular space between adjacent cells and regulate the solute movement across epithelial

sheets. TJs between neighboring cells allow the separation of apical and basolateral membrane domains that vary in protein and lipid contents, resulting in the maintenance of cell polarity and the regulation of cellular proliferation (19). An important hallmark of malignant transformation is loss of epithelial polarity (20).

In normal polarized epithelial cells, the mitotic spindles are parallel to the epithelial planar axis, and cellular proliferation is regulated by intercellular contact. Cell-cell adhesion provides a cortical planar cue to orient the mitotic spindle parallel to the epithelial plane (21-23). In an early stage of epithelial tumorigenesis, the orientation of mitotic spindles becomes deregulated, resulting in out-of-plane division (24) (Figure 2). Cell division is controlled by cytoskeletal dynamics (i.e., the mitotic spindle, actin filaments and microtubules), and cell-cell adhesions and their associated molecules are thought to be connected to the cytoskeletal organization and partly control these cytoskeletal elements (22). Thus, misorientation of the cell division axis might cause changes in the function and localization of the cell-cell adhesion system.

Despite the lateral localization of TJs and their components in normal epithelial cell sheets, TJs and their components might be exposed on the cellular surface by the rotation of spindles, indicating that TJ components might be a therapeutic target during the early stage of epithelial tumorigenesis. Our goal is to develop a novel tumor-targeting therapy using the TJ components that are exposed to the apical membrane from the lateral membrane by rotation of the mitotic axis.

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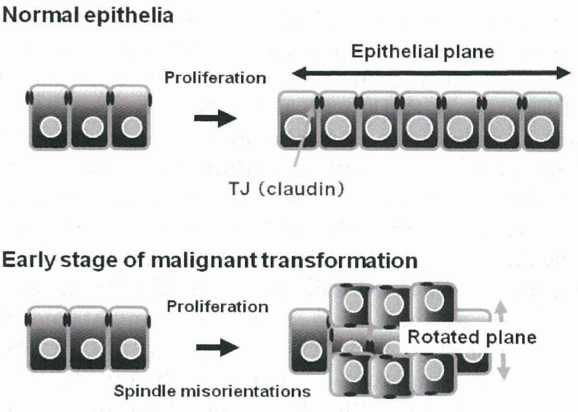

Figure 2. Scheme of malignant transformation in epithelium. In normal epithelial cells, the cells are divided in parallel with the epithelial plane. In the early stage of epithelial tumorigenesis, the orientation of the mitotic spindles is deregulated, and the cells proliferate by out-of-plane division (24).

Conflicts of Interest

No potential conflicts of interest to disclose.

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≪若手研究者紹介≫

生体バリアの分子基盤を利用した創薬研究

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1. はじめに

オーストラリアの動物学者ローレンツは，動物の生活史のある特定の時期に特定の物事がごく短時間で覚えこまれその記憶が長期間に渡り持続する学習現象「刷り込み」を見出している．小職の研究における刷りこみは，学部3回生の時に恩師の生物薬剤学の講義の中で聞いた「細胞製剤：細胞を敬え，細胞に学べ」という言葉である．本稿では，恩師による刷り込みを経て生体バリアの分子基盤を利用した創薬研究に至るまでの経緯を振り返ってみたい．

入学後テニスに明け暮れていた小職は3回生に進学してから，少しずつ研究室配属を考え始めるようになっていた．小職なりに「未来の薬」について思案してみたものの，キックサーブやトップスピンロブに関する知識では薬学に関するアイデアが思い浮かぶはずもなかった．小職なりに行き詰まりを感じていた矢先，恩師の生物薬剤学の講義の中で,「細胞製剤」という言葉を聞き，全身から鳥肌が立つような感動を覚えた．恩師は講義の中で,「自分の置かれた「場所」と「時間」に適合しつつ恒常性を維持している細胞が究極の剤形であること，この細胞から学ぶことにより必要な時に，必要な場所に，必要な量だけ薬を運ぶDDS技術の開発が可能になること」を熱く語られていた．大学院から恩師の研究室に入り,「細胞製剤：細胞を敬い，細胞に学ぶ」という学問を徹底的に叩き込まれた．細胞生物学に立脚した薬剤学を志向する研究生活を送る中で，同じサイトカインの刺激であっても細胞によって細胞内シグナル伝達経路が異なり，その結果として刺激に対して相反する反応が惹起される場合もあることを知り，細胞によって顔ばかりでなく細胞内も異なることに非常に興味を惹かれた．その当時の私の知識では，当時の薬剤学は細胞表面の議論に終始しているように思え（後に単なる勉強不足であることが分かったが），『1）細胞内の物質輸送の様子を見る，2）細胞の時間（細胞周期）を学ぶ，3）細胞内でのシグナル伝達の様子を見る』という3点について基礎力を身に付けた上で最終的に『独自の細胞製剤を開発する』という戦略を立て，博士課程在学中に理化学研究所抗生物質研究室に国内留学し，細胞周期阻害剤に関する研究に従事した．その後，徳島文理大学薬学部において，重金属や酸化的ストレスに対する生体応答反応に関する研究に携わり分子・細胞・個体レベルでの研究実施方法に関する基礎を多くの研究者からご教示頂き，同年代の研究者と「研究のオリジナリティーとは？」というテーマを肴に酒席において口泡を飛ばす議論を繰り返した．このときの議論仲間は，医学部，農学部，理学部，工学部など研究文化の異なるモザイク集団であった．振り返ると，この議論が小職の研究哲学をある程度形づくっていたように思う．

そして2002年4月に昭和薬科大学薬剤学教室に移動になったのを機に，独自の創薬研究領域の立ち上げに着手することを決意した．

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2. これまで

独自の創薬研究領域の立ち上げを決意した時に思い浮かんだ言葉が,「細胞を敬え，細胞を学べ」という恩師の教えであった．小職がこの世界に飛び込むきっかけとなったこの言葉を念頭に置き，まず「どの細胞から学ぶか？」ということ考え，①創薬研究において重要な細胞であること，②創薬研究領域において未開拓の分野が残されていること，③活用する細胞生物学的知見が国産であること，④温故知新という基本方針を設定した上で，細胞生物学の教科書を紐解き，上皮細胞に注目することにした．

周知のように上皮細胞は生体内外・組織内外を隔てるバリアとして機能していること，悪性腫瘍の90%が上皮由来であること，多くの病原性微生物の侵入門戸となっていることから，上皮細胞は薬物吸収，癌治療や感染症治療・予防における創薬ターゲットとして有用な性質を有している．上皮細胞を標的とした創薬研究としては，80年代より吸収促進剤が研究開発されているものの，実用化された吸収促進剤はカプリン酸ナトリウムのみであり，非特異的な物質の流入に起因する安全性確保が困難であることからparacellular routeを介した薬物吸収促進法の限界が指摘されている部分もあった．上皮細胞生物学の分野では，既に60年代にはtight junction（TJ）によってparacellular routeがシールされていることが知られていたが，TJ分子基盤の解明は遅れており，ようやく98年になってTJシールが膜蛋白質によって構成されていることが証明された．このことは，従来の吸収促進剤の限界はparacellular routeを利用した創薬研究の限界を示しているのではなく，上皮細胞生物学の遅延によりTJの分子基盤に立脚した創薬戦略が取られてこなかったことに起因していることを意味していた．そこで小職のグループでは，TJの分子基盤に立脚した創薬研究領域の開拓を広義の目標として設定し，TJシールの機能本体でありシール機能に組織特異性を有する分子という条件により標的分子の絞り込みを試み，京大月田グループにより同定されていたclaudinに注目した．

Claudinは分子量～23 kDaの4回膜貫通蛋白質であり，24種類の分子からなるファミリーを形成している．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は皮膚バリア，claudin-5は血液脳関門バリアを担っている．さらに，ヒトでは12種類の癌において発現異常が認められること，粘膜免疫組織に高発現していること，ウイルスの感染受容体としても機能していることから，claudinを標的とした薬物吸収促進法，癌ターゲティング法，粘膜ワクチン，抗ウイルス薬の開発など，新たな創薬研究領域創成の可能性が強く示唆された．しかしながら，claudinは抗原性が低い上に立体構造が解析されておらず，抗体を含めてclaudin binderの創製は著しく立ち遅れており，claudinを利用した創薬研究は皆無に等しいのが現状であった．

ウエルシュ菌下痢毒素（CPE）はヒトの食中毒を引き起こすことから細菌学の分野で詳細な解析が進められており，97年にCPE受容体が同定されていたものの，本受容体の生理的役割についての解析は遅々として進展していなかった．99年月田グループにより，CPE受容体がclaudin-4であること，CPEの受容体結合領域断片（C-CPE）が細胞傷害性を伴うことなくclaudin-4に結合しclaudin-4バリア機能を阻害することが報告され，claudinがTJバリア機能を担っていることが実験的に初めて証明されていた．小職は，徳島文理大在職中にウエルシュ菌研究の世界的権威である櫻井純先生から，細菌毒素の持つ特異性について薫陶を受けていたこともあり，この月田グループの報告を読んだ時にC-CPEを利用することでclaudinを標的とした創薬研究を展開できると直感した．さらに，C-CPEはポリペプチドであり将来的な遺伝子工学的手法を用いた改変が容易（C-CPEをprototypeとして用いた新規claudin binderの創製が可能）であることから，C-CPEをclaudin binderのモデル分子として利用し，claudinを利用した創薬研究に着手することにした．

2002年7月に，C-CPEの遺伝子を持っている阪大微研堀口先生に手紙を差し上げ，C-CPE cDNAを譲渡して頂き，試行錯誤しながらC-CPE蛋白質を作製した．そして，当時修士1年生であった浅野長祥君にお願いし，モデル薬物として分子量4000のデキストラン（FD-4）を用いてラット腸管ループ法により粘膜吸収促進活性を解析してもらった．すると，わずか0.2 mg/mlの処理で劇的なFD-4の吸収促進活性が観察され，このとき粘膜傷害性は観察さ

れなかった．当時小職は天然物由来の生理活性物質の機能解析および胎盤における亜鉛代謝に関する研究を進めており，claudinを利用した研究はエフォートの10%程度を割いているに過ぎない状況であった．実際，生理活性物質の機能解析については論文1報がアクセプトされ，複数の論文を投稿準備中という状況であり，両研究とも順調に推移していた．そこで，主に下級生を中心にテーマの選択と集中に関して忌憚の無いデスカッションを行い，両研究テーマを切り上げclaudinを利用した創薬研究にチームを挙げて取り組むことを決意した．そして，C-CPEが唯一のclaudin binderであることを踏まえ，①C-CPEをclaudin binderのモデル分子として利用し，claudinを利用した創薬研究の可能性を検証すること，②C-CPEをprototypeとして用いたclaudin binder創製系を構築すること，③新たなclaudin制御分子の探索を試みることをメインテーマとして設定し，claudinを利用した創薬研究に本格的にアタックすることにした．2002年12月のことである．

まず，C-CPEの粘膜吸収促進活性がclaudin-4を介して生じていることを確認するために，C-CPEのclaudin-4結合ドメインの同定を試み，C-CPEのclaudin-4結合領域欠損体を創出し，本欠損体を用いてclaudinを利用した粘膜吸収促進法の有用性を明らかにした（図1）[1]．さらに，C-CPE変異体を用いてclaudinを利用したペプチド医薬の経肺・経鼻吸収促進法を開発した[2]．また，C-CPEと緑膿菌エキソトキシン由来の蛋白合成阻害因子（PSIF）との融合蛋白質C-CPE-PSIFを作製し，claudin-4発現癌細胞に対する抗腫瘍活性を解析し，C-CPE-PSIFがclaudin-4指向性分子であること，C-CPE-PSIFが*in vitro*および*in vivo*で抗腫瘍活性を有することを見出し，C-CPEを用いた癌ターゲテイング法を確立した（図2）[3,4]．さらに，2003年に東大医科研の清野グループにより，粘膜免疫組織にclaudin-4が高発現していることが見出され，claudin-4を標的とした粘膜免疫組織への抗原デリバリーの可能性が示唆されていた．そこで，C-CPEとモデル抗原の融合蛋白質を作製し，claudin-4を標的とした粘膜ワクチン創製の可否を検証し，C-CPEとモデル抗原との混合液投与では抗原特異的な免疫応答が観察され

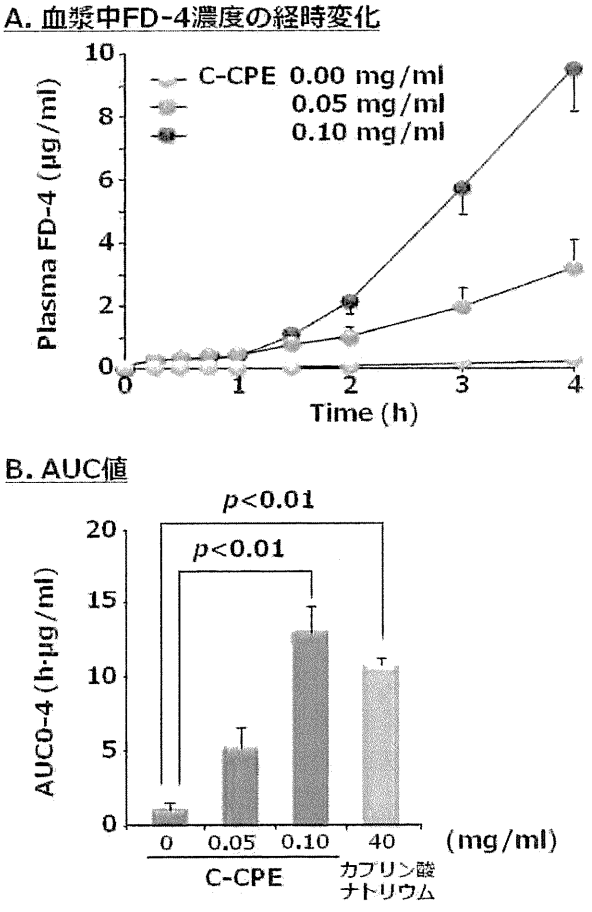

図1　C-CPEの腸管吸収促進活性
FITCラベルした分子量4000のデキストラン（FD-4）をモデル薬物として用いて，ラット腸管ループ法によりC-CPEの吸収促進活性を解析（文献1を一部改変）．

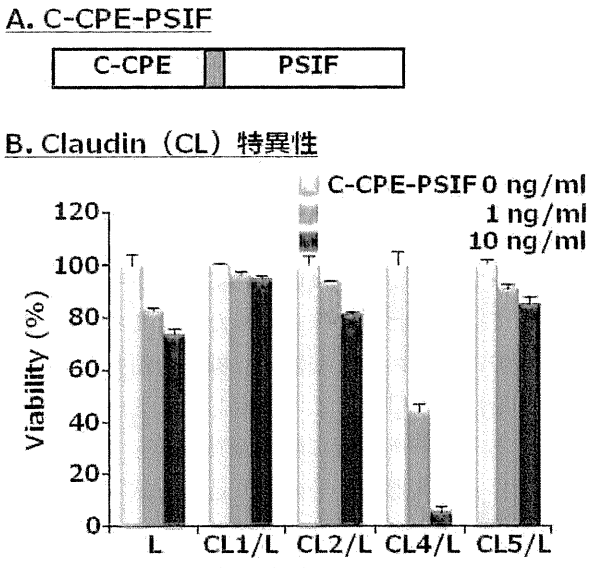

図2　Claudin-4指向性分子
Pseudomonas aeruginosa exotoxinの蛋白質合成阻害ドメイン（PSIF）とC-CPEとの融合体（A）を作製し，各種claudin発現細胞に各濃度24時間処理後にWSTアッセイにより細胞毒性を解析（B）．

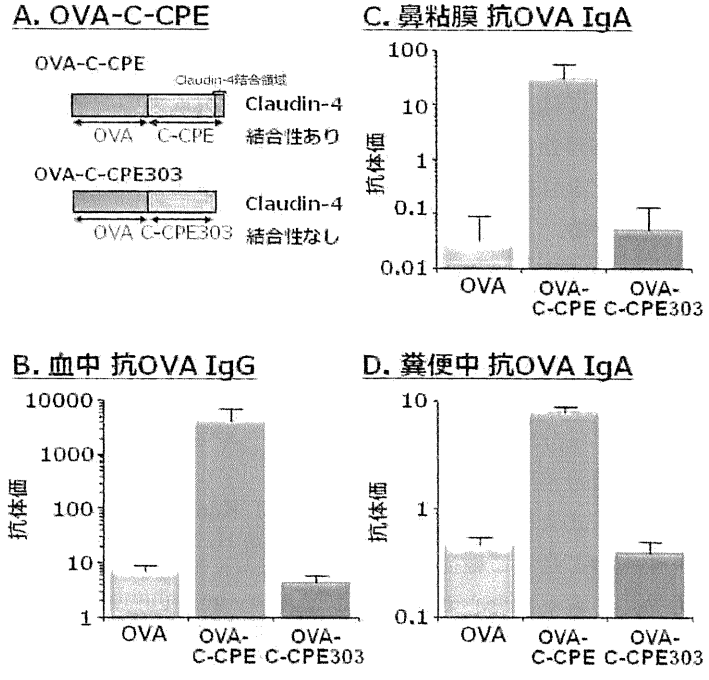

図3　Claudin binderを利用した粘膜ワクチン
モデル抗原（卵白アルブミン：OVA）とC-CPEとの融合体（A）を作製し，マウスに経鼻投与し，血中(B)，鼻粘膜洗浄液(C)，糞便抽出液(D)に含まれるOVA特異的抗体価を解析．

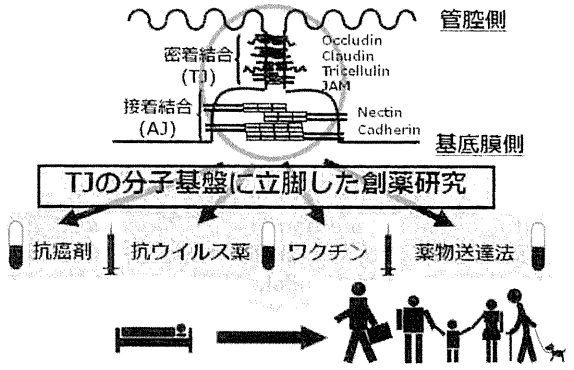

図4　生体バリアを利用した創薬研究

ないこと，融合蛋白質投与によって投与粘膜面のみならず遠隔粘膜面での抗原特異的IgA産生が起きること，claudin-4結合性が消失したC-CPE変異体との融合蛋白質では抗体価の上昇が観察されないことを見出し，claudin-4を標的とした粘膜ワクチン技術を初めて確立した（図3）[5]．さらに，C-CPEをprototypeとして用いたclaudin binder創製を図るために5年余りの歳月をかけて，C-CPEのclaudin結合残基を網羅的に解析，claudin binderのスクリーニングシステムを構築し，最近新規claudin binderの取得に成功した（Unpublished data）．

以上，現在までの検討により，claudinを標的とした粘膜吸収促進法，癌ターゲテイング法，粘膜ワクチン技術を創製し，claudinを利用した創薬の可能性を先駆けて報告してきた．さらに，C-CPEに比して優れた粘膜バリア制御活性を有する新規claudin binderの創製，およびclaudin binder創製系の構築にも成功している．

3. これから

上述したように，現在までにclaudinが創薬ターゲットとして多くの可能性を秘めていることを見出してきた．Claudinは24種類の分子が多種多様な組み合わせによって様々な性質を有するTJシールを構成し，生体内の多種多様な内部環境を維持していることから，この内部環境維持機構を自由自在に制御することができれば，新たなdrug delivery system（DDS）の開発に繋がる可能性がある．今後は，独自のclaudin binder創製技術を駆使して，多種多様な結合域を有するclaudin binderを創製し，組織特異性および透過物質特異性を併せ持つ新規薬物吸収促進法の開発，新規癌ターゲテイング法，ウイルス感染阻害剤の開発などの創薬研究を展開していきたい（図4）．

三つ子の魂百までというように，「細胞を敬え，細

胞に学べ」という刷り込みに支配され，上皮細胞およびclaudinの可能性に魅せられ，現在まで歩んできた．引き続き小職らのグループでは，本邦独自の細胞生物学研究土壌に育まれた本邦独自の創薬領域の開拓，およびその成果の実用化を目指し，激烈にチャレンジを続けていく予定である．

本稿を執筆する機会を与えて頂いた出口芳春先生を始めとした編集委員の先生方に深謝申し上げます．また，本稿でご紹介した研究成果は，大阪大学大学院薬学研究科生体機能分子化学分野八木清仁先生，昭和薬科大学薬剤学研究室渡邉善照先生，藤井まき子先生，アスビオファーマ株式会社内田博司先生をはじめとした多くの先生方のご指導ご鞭撻の所産であり，相互作用頂いた全ての方々に衷心よりお礼申し上げます．

また，実際の研究は多くの優秀な学生によって進められております．Claudinを利用した粘膜吸収促進に関する研究は増山茜さん，原田東樹君，高橋梓さん，古宮栄利子さんを中心としたグループ，claudinを利用したバイオ医薬の非侵襲性投与法の開発は山浦利章君，高橋梓さん，松久幸司君，松下恭平君，各務洋平君を中心とした研究グループ，claudinを利用した癌ターゲティング法の開発は海老原千晶さん，佐伯理恵さん，角谷秀樹君を中心とした研究グループ，claudinを利用した粘膜ワクチンの開発は角谷秀樹君，深坂昌弘君，鈴木英彦君を中心とした研究グループ，新規claudin binderの創製は高橋梓さん，山浦利章君，斎藤郁美子さん，松下恭平君，各務洋平君を中心とした研究グループによって進められたものでございます．この場をお借りして相互作用頂いた学生の皆様に改めてお礼申し上げます．

当該研究成果の一部は，文部科学省科研費，文部科学省知的クラスター創成事業，厚生労働省科研費，武田科学振興財団からのサポートにより実施されたものでございます．グラントサポートを賜りました関係者の皆様方に深謝申し上げます．

最後に，「細胞製剤」という学問を授けていただいた恩師眞弓忠範先生に衷心よりお礼を申し上げ，拙稿の結びとさせていただきます．

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A Claudin-Targeting Molecule as an Inhibitor of Tumor Metastasis[S]

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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,

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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.

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 never been applied into a ligand for claudin CPE receptors because of its strong cytotoxicity. The receptor-binding region of CPE (C-CPE) can be used for claudin-targeted cancer therapy (Saeki et al., 2009). Immunotoxins, consisting of a protein toxin connected to a binding ligand, such as an antibody or growth factor, have been developed and used for clinical therapy. Protein synthesis inhibitory factor (PSIF) derived from *Pseudomonas* exotoxin is a widely used protein toxin (Kreitman and Pastan, 2006), and intratumoral administration of the claudin-4-targeting PSIF has been shown to attenuate solid tumor growth (Saeki et al., 2009). In the present study, we investigated whether claudin CPE receptors can be a target for tumor metastasis by using the claudin-4-targeting PSIF as a claudin-targeting agent.

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 mmol/ml HEPES, respectively. The cells were maintained in a 5% CO_2 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 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 anticlaudin-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) (2×10^4 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 (1×10^5 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 $MgCl_2$, 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). 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 1×10^4 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 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 C57/BL6 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 (5×10^5 or 1×10^6 cells) in 100 μl of PBS were injected intravenously into the tails of C57/BL6 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, 1×10^5 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 $a \times b \times b/2$, where a represents the minimal tumor diameter, and b represents the maximal tumor diameter. The body weights of mice were also monitored.

For antitumor activity in 4T1 cells, 4T1 cells (1×10^5cells) 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

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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 mm^3 in the vehicle-treated group and 740.5 ± 94.6 mm^3 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

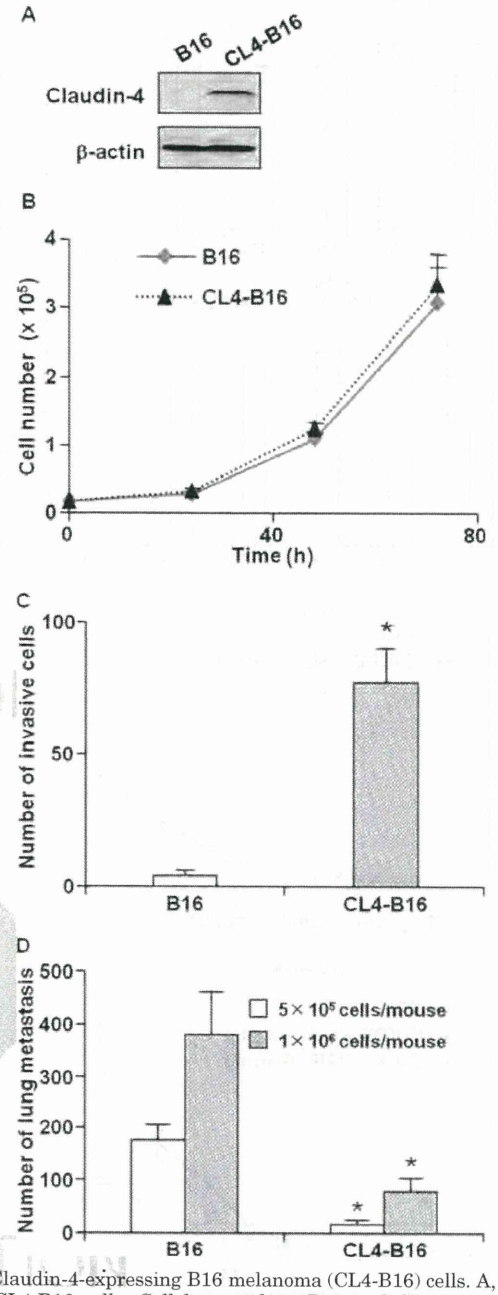

Fig. 1. Claudin-4-expressing B16 melanoma (CL4-B16) cells. A, preparation of CL4-B16 cells. Cell lysates from B16 and CL4-B16 cells were subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blotting with claudin-4 and β-actin. β-Actin is a control for an endogenous protein. B, the effect of claudin-4 on cellular proliferation in B16 cells. B16 or CL4-B16 cells (2×10^4 cells) were seeded onto a 24-well plate. Then, the cell numbers were counted by trypan blue dye exclusion assay at the indicated periods. Data are shown as means ± S.D. ($n = 4$). C, effect of claudin-4 on invasion in B16 cells. B16 or CL4-B16 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 ± S.D. ($n = 4$). *, significantly different from B16 cells ($p < 0.01$). D, lung metastasis of CL4-B16 cells. B16 or CL4-B16 cells (5×10^5 or 1×10^6 cells) were injected into the tail veins of mice. After 14 days, the mice were sacrificed, the 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 ($p < 0.01$).

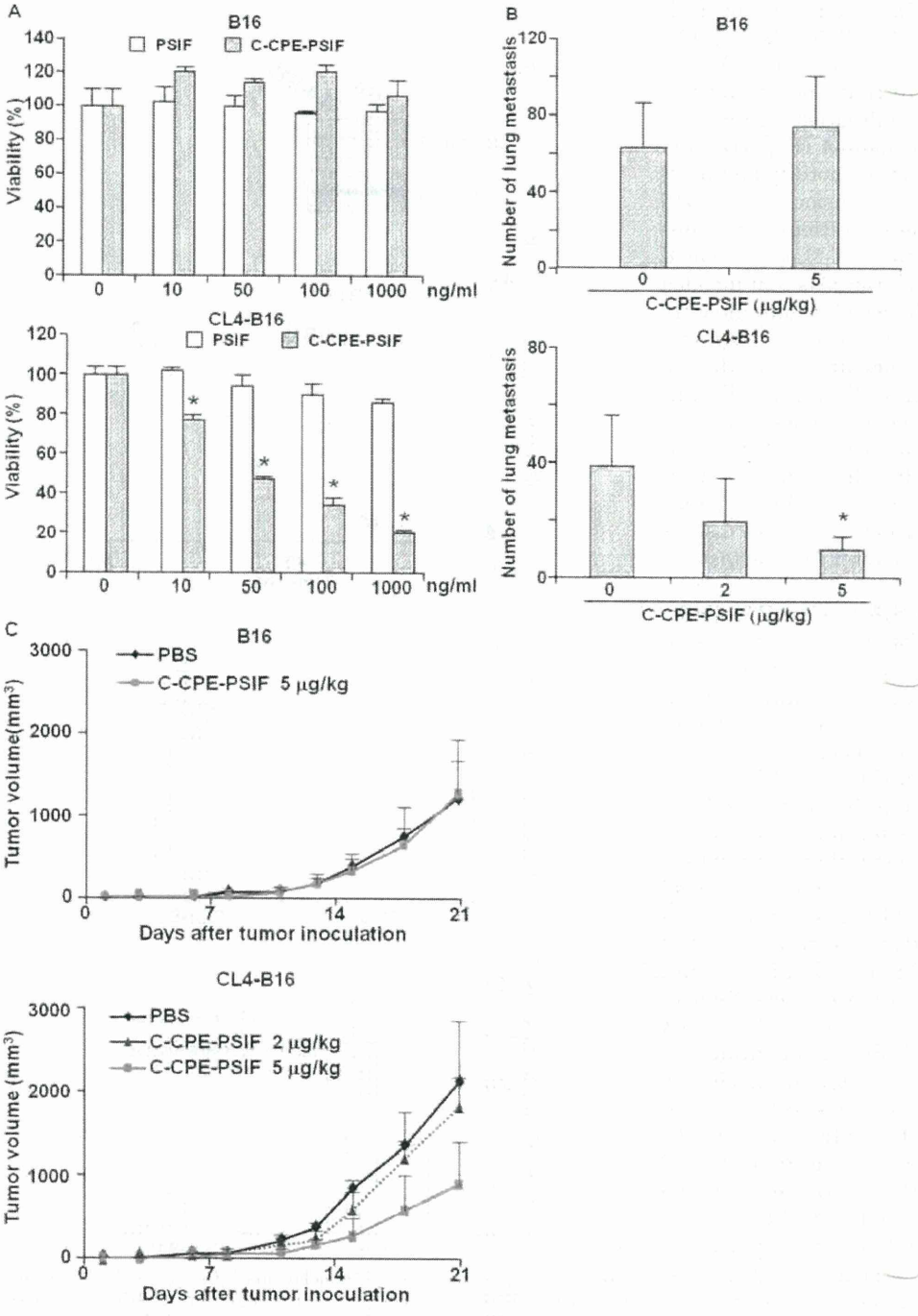

Fig. 2. Antitumor activity of C-CPE-PSIF for CL4-B16 cells. A, cytotoxicity of C-CPE-PSIF in CL4-B16 cells. B16 cells (top) and CL4-B16 cells (bottom) were treated with PSIF or C-CPE-PSIF at the indicated concentrations for 24 h. Cell viability (%) was measured by a WST-8 kit, according to the manufacturer's instructions (Nacalai Tesque). Data represent the mean ± S.D. ($n = 3$). *, significantly different from the vehicle-treated group ($p < 0.05$). B, antimetastatic activity of C-CPE-PSIF on lung metastasis of B16 (top) or CL4-B16 (bottom) cells. B16 or CL4-B16 cells (1×10^6 cells) were injected into the tail veins of mice on day 0, and vehicle or C-CPE-PSIF (2 or 5 μg/kg) was intravenously injected on days 0, 2, 4, 7, 9, 11, and 13. On day 14, 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 the vehicle-treated group ($p < 0.05$). C, antitumor activity of C-CPE-PSIF on CL4-B16 subcutaneously inoculated allograft. B16 (top) or CL4-B16 (bottom) cells (1×10^5 cells) were intradermally inoculated into the right flank of mice on day 0, and PBS or C-CPE-PSIF (2 or 5 μg/kg) was intravenously injected three times a week. Tumor volume was monitored. Each point is the mean ± S.D. ($n = 5$). The data are representative of two independent experiments.

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 mm^3 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.