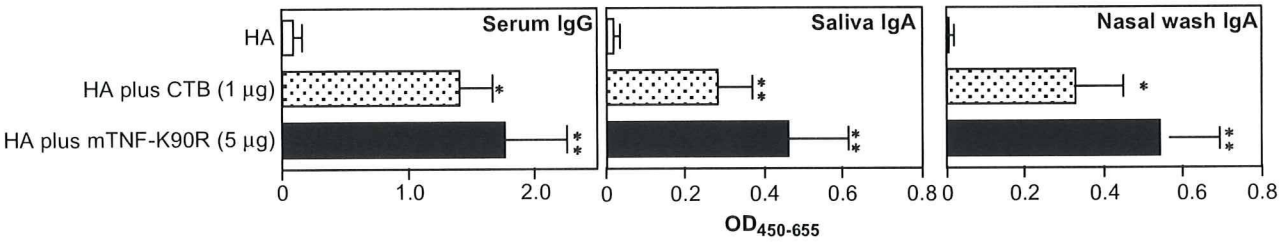

Fig. 5. mTNF-K90R induced mucosal IgA and IgG responses against influenza virus HA in mice. BALB/c mice were immunized intranasally with HA together with 1 μg CTB or 5 μg mTNF-K90R. One week after the last immunization, HA-specific IgG in serum at a 1:500 dilution and IgA in nasal or saliva at a 1:8 dilution were assessed by ELISA at a 1:8 dilution. Data represents the mean of absorbance 450 nm (reference wavelength, 655 nm). N.D; not detected. Data are presented as means ± SEM ($n = 4$–6; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* value for HA alone treated group by ANOVA).

due to the presence of a specific receptor, GM1 ganglioside, which is highly expressed in neuronal tissue [20]. This neurotoxicity has severely restricted the use enterotoxins as adjuvants for mucosal vaccines in clinical practice. To evaluate the *in vivo* toxicity of mTNF-K90R, inflammatory response and tissue injury were assessed in the nasal tissue (Fig. 7). Tissue sections of the nasal cavity were prepared at various time points after immunization of OVA plus mTNF-K90R. However, no tissue injury could be detected in any of the sections. These observations indicate no membrane barrier disruption and/or inflammatory changes, not even after 2 h single immunization with 5 μg mTNF-K90R (Fig. 7A and B). Furthermore, increasing the dose of mTNF-K90R given intranasally from 1 μg to 25 μg did not seem to have an adverse effect on the mice after three immunizations (Fig. 7C and D). Thus, although further evaluation is required, the results of this initial study demonstrated that the toxicity of mTNF-K90R is likely to be relatively low.

4. Discussion

In this study, we examined the mucosal adjuvant activity of mTNF-K90R and showed that intranasal co-administration of mTNF-K90R with antigen strongly induced both antigen-specific IgG in serum and IgA at mucosal site (nasal cavity, oral cavity, vagina and intestine). The enhanced adjuvant effect of mTNF-K90R might be caused by improved bioactivity and protease resistance compared to wTNF-α. Although mTNF-K90R showed a potent adjuvant effect on mucosal immunity, it does not elicit excessive inflammatory symptoms, such as formation of edema or fibrosis. Therefore, we believe that mTNF-K90R is a potent mucosal adjuvant for vaccines against various infectious diseases.

The development of a safe and effective vaccine is critical in preventing the spread of influenza virus. It is generally thought that or anti-influenza virus-neutralizing antibody must be induced at mucosal surfaces in order to prevent influenza virus infection. Previous studies also reported that antigen-specific systemic IgG and mucosal IgA Abs have potentially important roles for protection against the influenza virus [21,22]. Therefore, our results suggest that mTNF-K90R might be a superior mucosal vaccine adjuvant against infectious diseases caused by influenza virus.

TNF-α is anticipated to be used as a therapeutic drug to treat cancer. TNF-α has been used clinically for the treatment of non-resectable high-grade sarcoma and melanoma by locoregional applications in combination with melphalan under the approval of the European Agency for the Evaluation of Medicinal Products because systemic administration of TNF-α at therapeutically effective doses is limited by its unacceptable toxic side effects [23,24]. Further, in a recent report, it has been suggested that TNF-α had the potentials of the genetic toxicity, because TNF-α caused DNA damage through its ability to induce reactive oxygen species [25]. Thus, it is important to examine the safety of mTNF-K90R in a protocol using a mucosal vaccine adjuvant. Previously, we reported that mTNF-K90R had 1.3-fold lower *in vivo* toxicity after systemic administration compared with that of wTNF-α because of a change in the pharmacokinetics. Similarly, no adverse effect on the nasal mucosa was observed in this study after intranasal administration of mTNF-K90R. Although detailed examinations are required, mTNF-K90R is expected to be a useful mucosal vaccine adjuvant. Furthermore, we have shown that conjugating cytokines with polyethylene glycol (PEG) improves their safety *in vivo* [17,18,26–28]. We have also developed a novel site-specific PEGylation process to overcome the

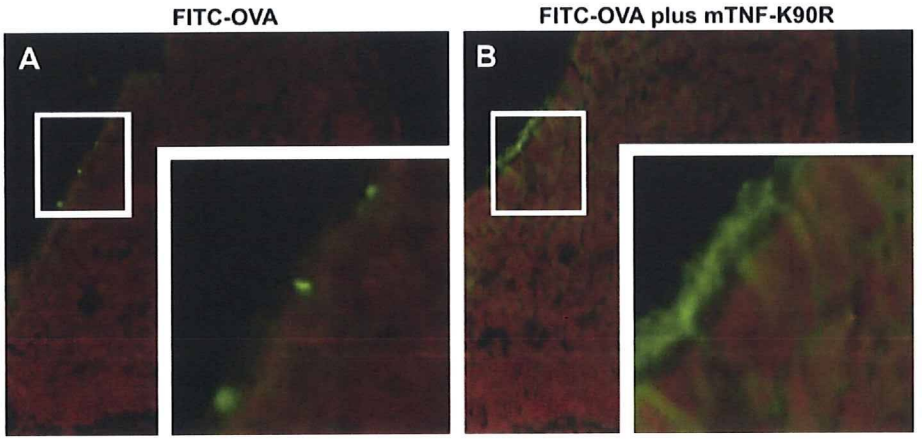

Fig. 6. Localization analysis of OVA in NALT. BALB/c mice were administered 50 μg FITC-OVA and a combination of 5 μg mTNF-K90R as a nasal vaccine adjuvant. Frozen sections of NALT resected from mice treated with FITC-OVA alone (A) and a combination of mTNF-K90R (B). The FITC-OVA (green) signals were detected by fluorescence microscopy. The nucleus was counterstained using PI (red). The original magnification of these photographs was 20×.

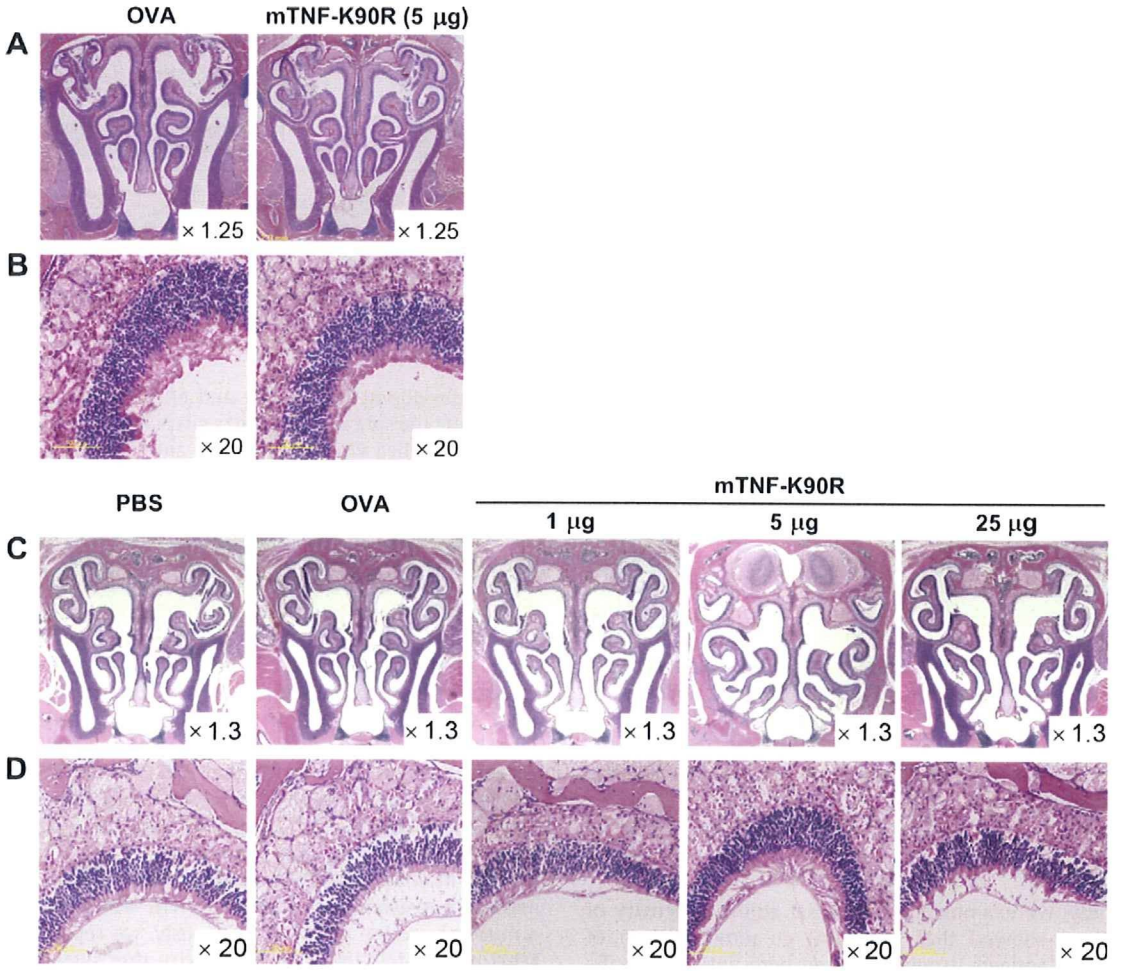

Fig. 7. Histopathological analysis of nasal cavity treated with mTNF-K90R. Frontal cross-sections of the nasal cavity from mice, taken 2 h after administration (A, B) or one week after three times administration of PBS, OVA alone, and OVA together with 1 μg, 5 μg or 25 μg mTNF-K90R (C, D). An overall view of the nasal passage is shown in (A) and (C). The region of nasal olfactory epithelia is shown in (B) and (D). Sections were prepared and the tissues were stained with H&E to assess the degree of tissue injury and local inflammation.

problems of PEGylation [17,18,26,28]. Previously we showed that the application of this technology to mTNF-K90R improved the safety and the anti-tumor effects of mTNF-K90R [18]. We are currently examining the safety and efficacy of site-specific PEGylated mTNF-K90R as a mucosal vaccine adjuvant.

The effects of mTNF-K90R at mucosal tissue was also analyzed. We reasoned that the adjuvant effect of mTNF-K90R may be related to its stimulation of antigen-presenting cells, such as DC. Indeed, DC plays a crucial role in eliciting T cell-dependent immunity. TNF-α is known to have profound effects on DC function and contributing to their activation, maturation and migration to, and accumulation within, draining lymph nodes [29,30]. Moreover, DC stimulated by TNF-α prior to anti-tumor vaccination or transfection with the TNF-α gene are reported to induce anti-tumor immunity [14,15]. However, we found that mTNF-K90R significantly enhanced the permeability of the nasal epithelial layer and diffusion of antigen into NALT. Consistent with our results, some reports have shown that TNF-α causes an increase in intestinal permeability [25,31]. Taken together, these results suggest that the strong mucosal adjuvant activity of mTNF-K90R is caused, at least in part, by increased epithelial permeability. In addition, TNF-α causes up-regulation of human polymeric Ig receptor on mucosal epithelial cells [32]. Polymeric Ig receptor transports polymeric IgA into external secretions as secretory IgA, which is critical for the defense of mucosal tissues [33]. We believe that multiple actions of mTNF-K90R contribute to its potent adjuvant activity. Currently, we are attempting to elucidate these various mechanisms.

Induction of both Th1- and Th2-type responses is the major goal for the development of mucosal vaccines because these responses would provide protective immunity against viral and bacterial infections by maximizing antigen-specific Ab and cytolytic T lymphocytes (CTL) responses. Although mTNF-K90R is not likely to induce CTLs, it could efficiently induce Abs responses. To induce both antigen-specific Ab and CTL responses, combinatorial administration of mTNF-K90R with another mucosal adjuvant, which can induce Th1-type immune responses, is applicable. We have already screened the TNF superfamily and other cytokines and succeeded in finding candidates that can effectively induce CTL at the mucosal site. The combinatorial effect of the cytokines and mTNF-K90R as a mucosal vaccine adjuvant is now under examination.

Recently, vaccine therapy has been attempted not only to combat cancer or viral infections but also for other diseases such as Alzheimer-type dementia. Schenk et al. demonstrated that intraperitoneal vaccination of β-amyloid peptide plus Freund's adjuvant to a murine Alzheimer's disease model resulted in a dramatic reduction of cerebral amyloidosis [34]. This therapeutic approach is clearly efficacious; however, the safety of this strategy is of paramount importance. In a clinical trial, approximately 6% of patients

administered a synthetic β-amyloid peptide plus adjuvant developed aseptic meningoencephalitis, most likely mediated by brain-infiltrating activated T cells [35,36]. This adverse effect seemed to be associated with the activation of Th1-type immunity by vaccination with β-amyloid peptide [37,38]. Nikolic et al. demonstrated that immunization capable of inducing Th2-type immunity predominantly constitutes an effective and potentially safe treatment strategy for Alzheimer's disease [39]. Therefore, our mTNF-K90R is a promising development in the establishment of an easy-to-use, efficacious, safe immunotherapy for Alzheimer's disease. However, further analyses are necessary in order to elucidate the Th2-dominant mechanism of mTNF-K90R.

5. Conclusions

In summary, our study showed that mTNF-K90R, engineered by using a phage display technique, induced two layers of protective immunity when administered intranasally with a vaccine antigen. Our results indicate that mTNF-K90R is a safe and effective mucosal adjuvant. Additionally, our technique of creating bioactive mutant cytokines might be an attractive generic approach for designing novel mucosal adjuvants.

Acknowledgements

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Appendix

Figures with essential color discrimination. Figs. 6 and 7 in this article may be difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.07.009.

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Expert Opinion

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2. TJ components and TJ modulators
3. Physiological barriers modulated by TJ modulators
4. Expert opinion

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healthcare

Tight junction modulator and drug delivery

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Recent progress in pharmaceutical technology based on genomic and proteomic research has provided many drug candidates, including not only chemicals but peptides, antibodies and nucleic acids. These candidates do not show pharmaceutical activity without their absorption into systemic flow and movement from the systemic flow into the target tissue. Epithelial and endothelial cell sheets play a pivotal role in the barrier between internal and external body and tissues. Tight junctions (TJs) between adjacent epithelial cells limit the movement of molecules through the intercellular space in epithelial and endothelial cell sheets. Thus, a promising strategy for drug delivery is the modulation of TJ components to allow molecules to pass through the TJ-based cellular barriers. In this review, we discuss recent progress in the development of TJ modulators and the possibility of absorption enhancers and drug-delivery systems based on TJ components.

Keywords: absorption enhancer, claudin, drug delivery, occludin, paracellular route, tight junction

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1. Introduction

Drug candidates, including chemicals, peptides, proteins, nucleic acids and their derivatives, can be efficiently identified by a combination of high-throughput technology and genome-based drug discovery. However, two steps are required for the clinical application of these drug candidates: movement of the molecules into the body and tissue through epithelial and endothelial cell sheets. These cell sheets regulate the movement of solutes between tissues within the body as well as between the outside and inside of the body.

Routes for passing of drug through the epithelial and endothelial cell sheets are classified into transcellular and paracellular routes (**Figure 1**). In the transcellular route, drugs are delivered by simple diffusion into the cell membranes and active transport via a receptor or transporter on the cell membrane [1,2]. Various transporters involved in the influx and efflux of peptides, organic anions and cations have been identified, and transcellular delivery systems using the transporters have been widely investigated [2-6]. Transporter-mediated drug delivery is tissue-specific and has low toxicity; however, the drugs must be modified for interaction with the transporter without loss of pharmaceutical activity. Thus, the transcellular route is not suitable for high-throughput production of drug candidates. The other route for drug delivery is the paracellular route. Tight junctions (TJs) seal the paracellular route and prevent the free movement of molecules in the paracellular space; therefore, a strategy for the paracellular delivery of drugs is the opening of TJs [7,8]. Compared with the transcellular route, the paracellular route has the advantages that drug modification is not needed and that one method can be applied for various drugs. Drug delivery systems through the paracellular route have been investigated as absorption enhancers since the 1980s. However, only sodium caprate is currently used as an absorption enhancer in pharmaceutical therapy.

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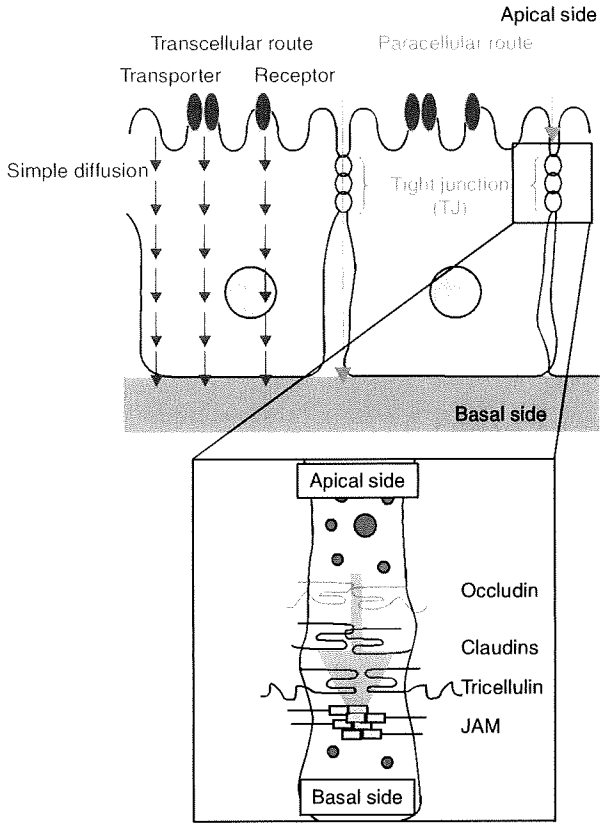

Figure 1. Schematic illustration of transport routes in epithelia.

It had been unclear how TJs regulated movement of solutes and what TJs were. In 1993, Furuse and colleagues determined that occludin, a protein with four transmembrane domains, is a component of TJs and that TJs consist of protein [9]. In 1998, Furuse and co-workers also identified another TJ protein, claudin-1 and -2 [10]. Claudins, a multigene family of at least 24 members, are key molecules of the TJ barrier [11]. Schematic biochemical machinery of TJs is shown in **Figure 1**, and modulation of the TJ components to allow drugs to pass through the paracellular route has been investigated as a novel strategy for drug delivery since the first report of TJ component-based drug delivery using an occludin peptide corresponding to part of the extracellular loop [12].

In this review, we examine recent topics in TJ-based drug delivery systems that use both approaches – TJ component/TJ modulator and TJ barrier – and discuss the future direction of such systems.

2. TJ components and TJ modulators

In the first section, we reviewed recent progress in TJ modulators over the past 2 years with respect to TJ components and modulators of TJ barrier.

2.1 Claudin

Claudin is a four-transmembrane TJ protein with a molecular mass of around 23 kDa, and comprises a family of at least 24 members [10]. Expression of each claudin member varies among cell types and tissues [13,14]. Claudins are thought to polymerize and form TJ strands in a homomeric and heteromeric manner, and the combination and mixing ratios of different claudin species determine the barrier properties of TJs, depending on the tissues [11]. For instance, deletion of claudin-1 causes dysfunction of the epidermal barrier [15], and deletion of claudin-5 causes dysfunction of the blood–brain barrier [16]. These findings indicate that a specific claudin modulator would be useful for tissue-specific drug delivery through the paracellular route. The C-terminal receptor binding region of *Clostridium perfringens* enterotoxin (C-CPE) is the only known modulator of claudin-4 [17]. Cells treated with C-CPE have decreased intracellular levels of claudin-4 as well as disrupted TJ barriers in epithelial cell sheets [17]. We previously found that the jejunal absorption-enhancing effect of C-CPE was 400-fold more potent than that of sodium caprate, the only clinically used absorption enhancer [18]. The development of other claudin modulators by using C-CPE as a prototype is a promising strategy. Deletion assays and site-directed mutagenesis assays indicate that the C-terminal 16 amino acids of C-CPE are involved in its modulation of claudin-4 and that Tyr residues at positions 306, 310 and 312 are critical for C-CPE activities [19,20]. Van Itallie and colleagues revealed that the structure of C-CPE is a nine-strand β sandwich and that the C-terminal 16–amino acid fragment is located in the loop region between the β8 and β9 strands, indicating that the claudin-4 binding site is on a large surface loop between strands β8 and β9 or on a domain containing these strands [21]. These findings indicate that peptides containing the loop structure formed by the β8 and β9 strands are likely to be novel claudin modulators. Considering the antigenicity of the claudin-4 modulator, smaller peptides are useful. Recently, the 12-mer peptide binders of claudin-4 were successfully identified using a random 12-mer peptide phage-display library [22]. The common claudin-binding motif <XX(Y/W)$(X)_{3\ or\ 4}$Y(Y/X)(L/I)XX> was also detected. The 12-mer peptide was bound to claudin with nanomolar affinity, but it did not modulate the claudin barrier. A 27-mer amino acid peptide corresponding to the extracellular loop region of claudin-1 modulated epithelial barrier through its interaction with claudin-3 [23]. Distinct species of claudins can interact within and between tight junctions [24]. Thus, a short peptide corresponding to the extracellular loop region of the heterotypically interacting claudin is also a candidate of claudin modulator.

2.2 Occludin

Occludin, a 65-kDa protein containing four transmembrane domains, was the first TJ-associated integral protein to be identified [9]. The initial strategy for TJ component-based

drug delivery was to use a synthetic peptide corresponding to the extracellular loop region of occludin *in vitro* [12]. The testes are rich in receptors for follicle-stimulating hormone. The effects of follicle-stimulating hormone-fused occludin peptide on the *in vivo* blood-testis barrier were investigated. The fusion protein modulated the blood–testis barrier, resulting in delivery of inulin into the testis [25]. Astrovirus infection causes diarrhea [26]. Moser and co-workers found that the astrovirus capsid disrupted occludin and increased the permeability of the TJ barrier without cytotoxicity in human intestinal cells [27]. A pro-inflammatory cytokine, IL-1β causes a functional opening of the intestinal TJ barrier without induction of apoptosis [28,29]. The IL-1β-induced enhancement of TJ permeability was mediated by downregulation of occludin through an increase in the myosin light chain kinase [29,30].

Thus, occludin peptides containing the ligand-targeting motifs and novel types of occludin modulators, such as the component capsid and the activator of myosin light chain kinase, may provide novel methods to deliver drugs into target tissues across endothelial cell sheets.

2.3 Ephrin

Ephrin-A2, a family of receptor tyrosine kinases, directly phosphorylates claudin-4 in epithelial cells, leading to the disruption of the epithelial barrier function [31]. Intravenous administration of ephrin-A2 ligand causes vascular permeability in the lungs, resulting in the leakage of albumin into the lungs of rats [32]. The ephrin-A2 ligand is altered in the disruption of the TJ barrier in the lungs of rats and in cultured lung vascular endothelial cells [32]. High levels of ephrin-A2 mRNA are also expressed in the intestine [33]. A modulator of the ephrin-A2 system will be a novel type of pulmonary and intestinal absorption enhancer.

2.4 Zonula occludens toxin

Zonula occludens toxin (Zot) is a 44.8-kDa envelope protein of *Vibrio cholera*, and zonulin is the intestinal Zot analogue that governs the permeability of intercellular TJs [34-36]. Zot and Zot derivatives are reversible TJ openers that enhance the delivery of drugs through the paracellular route without toxicity [35-40]. The Zots bind to a putative receptor on the apical surface of enterocytes, leading to protein kinase C-mediated polymerization of soluble G-actin and the subsequent loosening of TJs [38,41]. Zot enhanced the absorption of insulin in diabetic rats, and the bioavailability of oral insulin was sufficient to lower the serum glucose concentrations to an extent that was comparable to the parenteral injection of the hormone [35]. In 2001, an active fragment of Zot, ΔG with a molecular mass of 12 kDa, was identified [42]. In 2008, a hexapeptide derived from Zot, AT1002, was found to enhance absorption [43]. AT1002 increased permeability in human epithelial cell sheets without cytotoxicity and enhanced duodenal absorption of ciclosporin A.

2.5 Chitosan

Chitosan is derived from chitin, a polysaccharide found in the exoskeletons of insects, arachnids, and crustaceans. Chitosan is a nontoxic, biocompatible and mucoadhesive polymer that is a safe and efficient intestinal permeation enhancer for the absorption of drugs [44-46]. The chitosan-mediated activation of protein kinase Cα is followed by the redistribution of ZO-1 and an increase in TJ permeability, suggesting that the protein kinase Cα-dependent signal transduction pathway affects TJ integrity [47]. The oral administration of recently developed chitosan-coated nanoparticles containing insulin dramatically decreased blood glucose levels in diabetic rats [48].

2.6 HA, HAstV-1

Hemagglutinin (HA), a non-toxic component of the large 16S of the botulinum neurotoxin [49], and the human astrovirus serotype 1 (HAstV-1) capsid [27] may be a novel absorption enhancer via the paracellular route. The HA protein affected distribution of occludin, ZO-1, E-cadherin and β-catenin, and increased TJ permeability in human intestinal epithelial cells without cytotoxicity [49]. When HAstV-1 infected a Caco-2 cell monolayer from the apical side, the paracellular permeability was increased. UV-inactivated HAstV-1 also increased the permeability and disrupted occludin, indicating that the enhancement of the permeability was not dependent on viral replication [27]. Further analysis of the mode of action of these toxin- and virus-derived enhancers will lead to the development of novel intestinal absorption enhancers.

3. Physiological barriers modulated by TJ modulators

In the second section, we overviewed recent progress in TJ modulators with respect to the barrier separating different body compartments.

3.1 Blood–brain barrier

The blood–brain barrier, which comprises endothelial cell sheets with extremely tight junctions, limits the diffusion of hydrophilic molecules between the bloodstream and brain. Many pharmaceutical chemicals developed for the treatment of brain disorders cannot be applied in clinical therapy because they do not pass through the blood–brain barrier. Methods to open or reversibly regulate the blood–brain barrier have been investigated. Blood–brain barrier modulation based on the infection mechanisms of HIV has been proposed. Disruption of TJs occurs in the brains of HIV-infected patients [50-52], and tat protein, which is released from HIV-infected cells, decreases ZO-1 levels at the cell–cell borders in brain microvascular endothelial cells [53]. Tat treatment reduced expression of occludin, ZO-1, and ZO-2 in human brain microvascular endothelial cells via caveolin-1 and Ras signaling. Other HIV-1-derived proteins, gp120 and Nef,

Table 1. Candidates of absorption enhancer.

Target barrier	Candidates
Intestinal barrier	C-CPE
	AT1002
	Ephrin
	Chitosan and its derivatives
	Haemagglutinin
	HAstV-1 capsid
	Spermine
Blood-brain barrier	HIV-1 tat
	Sodium caprate
	Nitric oxide
Nasal barrier	AT1002
	Sperminated gelatin
	FDFWITP
Blood-testis barrier	C-type natriuretic peptide
	domain I of laminin β3

C-CPE: C-terminal of Clostridium perfringens enterotoxin; HAstv-1: Human astirovirus serotype 1; HIV: Human immunodeficiency virus.

can change the expression of TJ proteins *in vitro* [54]. Cocaine [55-56], sodium caprate [57] and nitric oxide [58] also modulate the blood–brain barrier.

3.2 Blood–testis barrier

Disruption of the blood–testis barrier affects spermatogenesis; thus, junctional proteins, such as occludin, ZO-1, and N-cadherin, could be the primary targets for testicular toxicants [59]. Monophthalates (mono-*n*-butyl phthalate and mono-2-ethylhexyl phthalate) were recently shown to disrupt the inter-Sertoli TJs in rat [60]. Phthalates are used as plasticizing and suspension agents in personal care products, plastics, paints, and pesticides. Monophthalates reduced the TJ barrier in Sertoli cells and induced the disappearance of ZO-1 and F-actin from around the cell periphery. The expression of occludin mRNA was also suppressed in a dose-dependent manner. C-type natriuretic peptide is a novel regulator of blood–testis barrier dynamics [61]. C-type natriuretic peptide regulates blood pressure, blood volume, fat metabolism, bone growth, and steroidogenesis in the testis and also reduces the expression of N-cadherin, occludin, and JAM-A [62,63]. Laminin fragments can also modulate the blood–testis barrier [64]. Treatment of primary Sertoli cells with domain I of laminin β3 caused a dose-dependent reduction in β1-integrin, occludin and ZO-1 and a decrease in the blood–testis barrier. Domain IV of laminin γ3 also reduced the expression of β1-integrin, occludin and JAM-A.

3.3 Epithelial barrier

Intranasal delivery is a convenient, reliable, rapid, and noninvasive delivery approach for low-molecular-weight compounds, and intranasal absorption enhancers have been developed for improvement of the nasal absorption of therapeutic macromolecules. AT1002, a polypeptide derived from Zot, enhanced not only intestinal absorption, but also nasal absorption of hydrophilic markers, PEG4000 and inulin [65]. Sperminated gelatin is a nasal absorption enhancer of insulin; when intranasally delivered, it decreases the plasma glucose level [66]. Aminated gelatin enhanced absorption of protein drugs through mucosal membranes with negligible mucosal damage [67].

Phage display technology is a powerful method for the selection of peptide ligands [68,69]. Recently, novel TJ modulators were isolated by using a phage-display library [70]. TJ-bound peptides were screened by using confluent monolayer cell sheets that were treated with a calcium chelator, EGTA. The polypeptide FDFWITP was isolated as a TJ binder. FDFWITP and its derivative peptides modulated TJ barriers without cytotoxicity, and these TJ-modulating activities were reversible. Thus, the phage-display system is a promising and powerful tool for developing TJ modulators.

4. Expert opinion

Many TJ-associated integral proteins, including occludin, claudin, tricellulin, ZO-1, ZO-2 and ZO-3, have been identified. These proteins play pivotal roles in the regulation of solute movement via the paracellular route, indicating that TJ modulators can be promising methods to deliver drugs. Studies of claudin-deficient mice initially indicated the possibility of TJ component-based drug delivery. Claudin-1-deficient mice lose their epidermal barrier function against a tracer with a molecular weight of around 600 Da, [15], indicating that claudin-1 modulators can enhance the transdermal absorption of drugs. The transdermal route is an easy, painless, and noninvasive method for drug administration, and the claudin-1 modulators have been the subject of pharmaceutical research. Claudin-5-deficient mice lose their blood–brain barrier [16], and small molecules (< 800 Da) selectively passed across the blood–brain barrier. The claudin-5 modulator will be a candidate for the pharmaceutical therapy of brain diseases. We found that the intestinal absorption-enhancing effects of a claudin-4 modulator were 400-fold more potent than those of a clinically used absorption enhancer [18]. Disruption of occludin or tricellulin increases TJ permeability [12,25,71]. These findings strongly indicate that modulation of TJ is a promising method for drug delivery. Because TJ proteins are poor in antigenicity, it is difficult to develop antibodies against the extracellular domain, resulting in a severe delay in the development of TJ modulators. At this point, there have been two breakthroughs in the development of TJ modulators. The first breakthrough is the determination of the structure of the only known claudin modulator, C-CPE [21]. The second breakthrough is the establishment of an efficient phage-display method to isolate a novel peptide to bind TJ components [22]. We believe that the

development of a claudin modulator by using C-CPE as a prototype will be successful, and that a peptide type of TJ modulator will be prepared in the near future. We are also optimistic about the production of a novel TJ modulator based on fragments of toxins, viruses and natural products. These fragments appear to use a novel mechanism to modulate the TJ barrier, and further analysis of this novel type of TJ modulator may lead to the next generation of TJ modulators (**Table 1**).

Very recently, Lee and colleagues proposed the lipid-protein hybrid model for TJ that the TJ proteins by themselves, and in combination with the lipids, serve, in addition, essential roles in barrier function, indicating that a lipid modulator can be a TJ modulator [72]. Glycosylated sphingosine, oxidized lipids and ether lipids were identified as TJ modulators, and the displacement of claudins and occludin from lipid raft was involved in the absorption-enhancing effect of sodium caprate [73,74]. Future investigation of the lipid-protein hybrid model for TJ may be the third breakthrough in the development of TJ modulators.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

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

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ABSTRACT

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

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

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

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

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

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

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

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

Materials and Methods

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Results

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

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

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

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

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

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

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

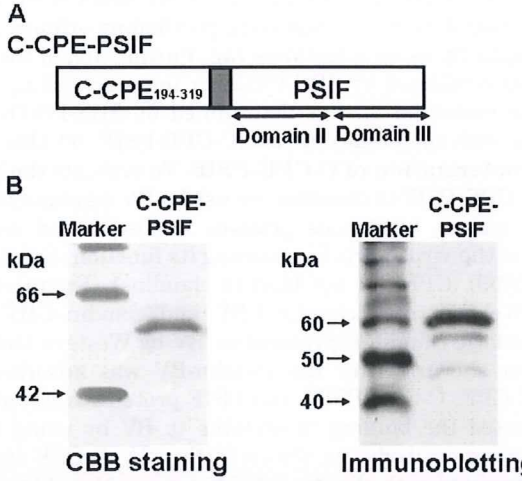

Fig. 1. Preparation of C-CPE-PSIF. A, schematic structure of C-CPE-PSIF. C-CPE-PSIF is a fusion protein of C-CPE and PSIF. C-CPE is the C-terminal fragment of CPE 194 to 319 aa (Van Itallie et al., 2008). The dark area indicates the putative receptor-binding region of C-CPE located in its C terminus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

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

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

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

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

Discussion

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

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

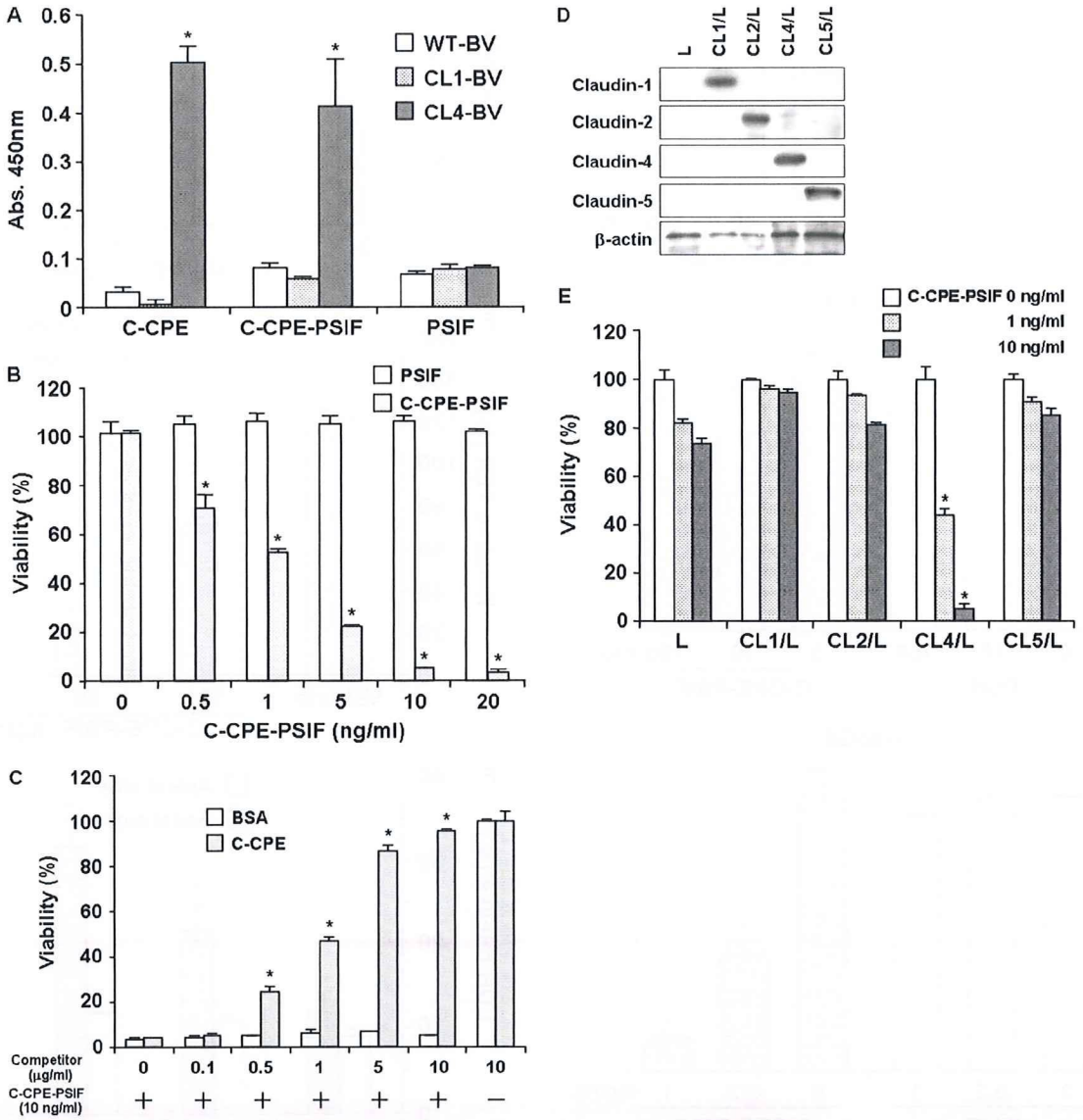

Fig. 2. Characterization of C-CPE-PSIF. A, interaction of C-CPE-PSIF with claudins. Wild-type BV (WT-BV), claudin-1-BV (CL1-BV), or claudin-4-BV (CL4-BV) was adsorbed onto a 96-well immunoplate at 0.5 μg/well. Then, 7.5 μmol C-CPE, C-CPE-PSIF, or PSIF was added to the well, and the protein bound to the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

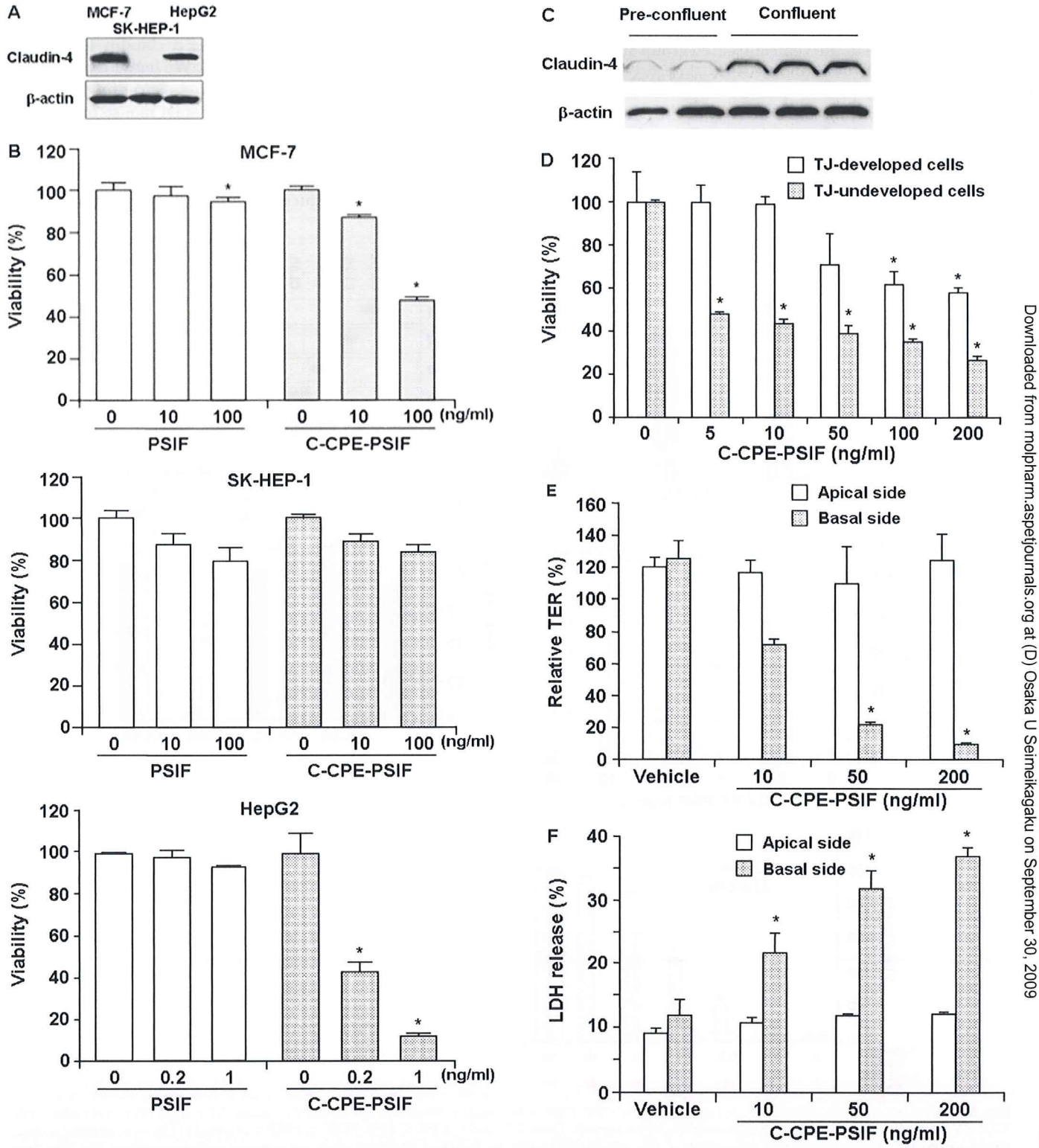

Fig. 3. Cytotoxic specificity of C-CPE-PSIF. A, expression of claudin-4 in SK-HEP-1 and HepG2 cells. The cell lysates were subjected to SDS-PAGE, followed by Western blotting with anti-claudin-4 Ab. MCF-7 cells were used as a positive control. B, cytotoxicity of C-CPE-PSIF in SK-HEP-1 and HepG2 cells.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

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

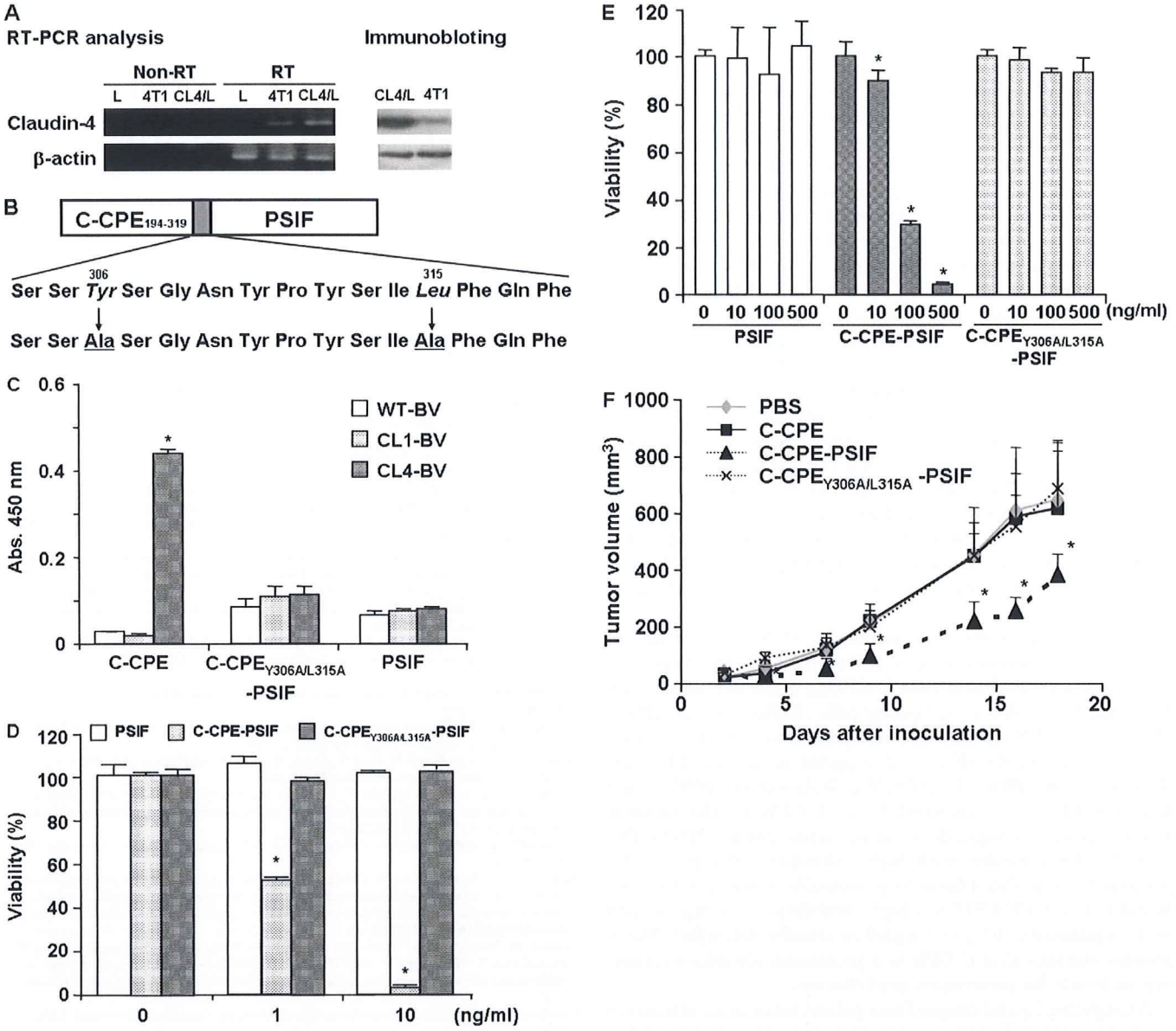

Fig. 4. In vivo antitumor activity of C-CPE-PSIF. A, expression of claudin-4 in 4T1 cells. Total RNA was extracted from the cells, followed by reverse transcription-polymerase chain reaction analysis (left). Cell lysates were subjected to SDS-PAGE, followed by Western blotting (right). β-Actin is an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

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

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

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

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

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TOPICS

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生体バリアを利用した創薬研究

第1回日本DDS学会奨励賞(臨床)受賞によせて

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

*Masuo Kondoh・Azusa Takahashi・Rie Saehi・Kiyohito Yagi**

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

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

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

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

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

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

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

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