

シャー 344 系ヌードラットの門脈から四塩化炭素 (1 ml/kg body weight) を週 2 回, 4 週間投与し肝傷害を与えた。分化誘導培地あるいは非誘導培地で培養後蛍光色素である PKH26 で染色し, 四塩化炭素初回投与 2 日後に門脈より 1×10^7 個の細胞を移植した。コントロールとしては四塩化炭素の替わりにオリブオイルを腹腔内投与したもの, 及び四塩化炭素を投与し細胞の替わりに生理食塩水を門脈から投与したもの (sham operation) を用意した。凍結肝臓切片を作成し蛍光観察を行った。細胞移植群においては誘導培地, 非誘導培地で培養した双方で生着が確認された。骨髄由来の間葉系幹細胞 (BMSC) を移植した際には個々の蛍光が散在していたが,²²⁾ 歯胚由来細胞移植の場合はコロニー状の像が観察されたことから生着後に増殖したものである。In vitro の培養において歯胚由来細胞は BMSC に比べ旺盛な増殖能を有しており, 生着後の増殖を可能にしたものと思われる。

分化誘導した細胞の移植群では有意に血清 AST, ALT 値の低下, 肝線維化の抑制が観察された。非誘導培地で培養した細胞を移植した群は肝臓内に生着していたにもかかわらず有意な治療効果は現われなかった。これらの結果より肝細胞への方向付けを行うことが重要であることが示された。²³⁾ このように破棄される組織から再生医療に有用な幹細胞が得られることは重要であり, 自己の親知らずを抜歯した際, 歯胚由来幹細胞を細胞バンクに保存しておけば自身の細胞を肝疾患の治療に利用することが可能となるであろう。また旺盛な増殖能があることから移植までのつなぎとしてパイオ人工肝臓へ利用することができること, 医薬品スクリーニングの評価系としても, 倫理的な問題を持つ ES 細胞, 強制的に未分化状態に回帰させた iPS 細胞由来のものに比べ有用であると考えている。

6. おわりに

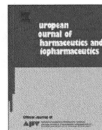
創薬の過程で毒性, 有効性, 及び薬物代謝の評価に大量の動物が用いられてきたが, 今後動物愛護の観点から in vitro の評価に置き換えていくことが求められている。肝細胞は毒性や薬物代謝を評価する際に重要な役割を果たすことは明らかであるがこれまで初代培養細胞の不安定性から有用な評価系は構築されていない。一般的に肝細胞のような足場依存性の細胞は培養表面の性状によりその生存性, 機能

が著しく変動することが知られている。本総説では dendrimer を用いる機能性培養基材の創製, 3 次元培養による機能維持効果, また遺伝子導入による寿命延長について筆者らの成果を紹介した。最小限の細胞で評価系を構築することに役立つ技術開発につながればと願っている。さらに通常廃棄されるヒトの組織から肝細胞を分化誘導し細胞源として用いることができれば倫理的問題, 種による差異を含め種々の問題点を解決できることになろう。

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Research paper

Claudin-4-targeting of diphtheria toxin fragment A using a C-terminal fragment of *Clostridium perfringens* enterotoxin

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ABSTRACT

Claudin (CL)-4, a tight junction protein, is overexpressed in some human neoplasias, including ovarian, breast, pancreatic and prostate cancers. The targeting of CL-4 is a novel strategy for tumor therapy. We previously found that the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) binds to CL-4. In the present study, we genetically prepared a novel CL-4-targeting molecule (DTA-C-CPE) by fusion of C-CPE and diphtheria toxin fragment A (DTA). Although DTA is not toxic to CL-4-expressing L cells, even at 20 µg/ml, DTA-C-CPE is toxic to CL-4-expressing L cells at 1 µg/ml. DTA-C-CPE-induced cytotoxicity was attenuated by pretreatment of the cells with C-CPE but not bovine serum albumin, indicating that DTA-C-CPE may bind to CL-4-expressing L cells through its C-CPE domain. To evaluate the specificity of DTA-C-CPE, we examined its cytotoxic effects in L cells that express CL-1, -2, -4 or -5. We found that DTA-C-CPE was toxic to only CL-4-expressing L cells. Thus, C-CPE may be a promising ligand for the development of cancer-targeting systems.

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1. Introduction

Chemotherapeutic agents target the intracellular metabolic processes or growth rates that are different between malignant cells and normal cells, and rapidly growing cancer cells are sensitive to chemotherapies [1,2]. But, progressive cancer cells with a decreased growth rate respond poorly to chemotherapy [3]. Radiation therapy affects both the tumor and the surrounding normal tissue. These conventional therapies cause DNA damage, leading to genomic instability and susceptibility to neoplastic mutations [4]. Cancer cells often overexpress surface proteins, including growth factor receptors or antigens [5]; thus, targeting cancer cells by using the surface proteins is a promising strategy for cancer therapy. Ligands for growth factor receptors and cytokine recep-

tors have been fused with fragments of bacterial toxins, such as *Pseudomonas* exotoxin and diphtheria toxin (DT) [3,6].

Tight junctions (TJs) form the apical junctional complex in epithelial cell sheets and play pivotal roles in the barrier of the epithelial cell sheets and the fence separating basal and apical components, such as receptors and transporters, on the membrane [7]. Epithelial TJs are dynamic structures that are modulated during neoplastic transformation [8]. The relationship between abnormal TJ function and epithelial tumor development has been suggested by earlier studies showing alterations in the TJ structures of epithelial cancers [9,10]. Loss of tight junction integrity may allow the diffusion of nutrients and other factors necessary for the survival and growth of the tumor cells [8]. Destruction of the fence function of TJs can lead to overproliferation of tumor cells [11,12]. If TJ components are exposed to the cell surface in cancer cells, they may be a promising target for cancer therapy.

Claudins (CLs) are key molecules in the formation of TJs; proteins in the 24-member claudin family contain four transmembrane domains [13]. CL-4 is frequently overexpressed in several neoplasias, including ovarian, breast, pancreatic and prostate cancers [12,14]. Thus, CL-4 may be useful as a target molecule in cancer therapy. CL-4 is a receptor for *Clostridium perfringens* enterotoxin (CPE), which is a single 35-kDa polypeptide that causes food poisoning in humans [15]. CPE exhibited anti-tumor activity

Abbreviations: C-CPE, the C-terminal fragment of *Clostridium perfringens* enterotoxin; DTA, diphtheria toxin fragment A; DTA-C-CPE, C-CPE-fused DTA; DT, diphtheria toxin; TJ, tight junction; CPE, *C. perfringens* enterotoxin; CL, claudin.

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in CL-expressing cancers, such as breast [16], ovarian [17] and pancreatic cancers [18]. They did not observe side effects from CPE treatment, indicating that a ligand for CL-4 may be a promising candidate for cancer-targeting therapy.

CL has very low antigenicity, and there are few antibodies to the extracellular region of CL. CPE is composed of N-terminal cytotoxic domain and C-terminal receptor-binding domain [15]. C-CPE is the C-terminal receptor-binding domain, and C-CPE is the first CL-4-binder [19]. In the present study, we prepared a CL-targeting agent (DTA-C-CPE) consisting of C-CPE coupled to a protein synthesis inhibitory factor, fragment A of DT [20]. DTA-C-CPE had CL-4-specific cytotoxicity; thus, C-CPE may be a promising ligand for the development of cancer-targeting systems.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulphophenyl)-2H-tetrazolium (WST-8) and phosphatase inhibitor cocktail were purchased from Nacalai (Kyoto, Japan). Protease inhibitor cocktail and anti- β -actin mAb were obtained from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-labeled antibodies were obtained from Chemicon (Temecula, CA). Anti-His-tag antibody was purchased from Novagen (Madison, WI). All other reagents were of research grade.

2.2. Cell culture

L cells, a mouse fibroblast cell line, and mouse CL-expressing L cells were kindly provided by Dr. S. Tsukita (Kyoto University, Japan). Cells were cultured in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FBS) at 37 °C.

2.3. Preparation of DTA-C-CPE

DTA (CRM45) cDNA was kindly provided by Dr. K. Kohno (Nara Institute of Science and Technology, Japan) [21]. The plasmids containing DTA fused with C-CPE were prepared as follows. DTA was amplified by polymerase chain reaction (PCR) with pTA-DTA as a template, a forward primer (5'-GCGGTACCATGGGCGCTGATGATGTGTG-3', *KpnI* site is underlined) and a reverse primer (5'-CCTTAATTAATCGCGTACGCGATTCTCG-3', *PacI* site is underlined). The resulting PCR fragments were subcloned into *KpnI*/*PacI*-digested pETH₁₀PER (kindly provided by Dr. Y. Horiguchi, Osaka University, Japan), and the sequence was confirmed (pET-DTA-C-CPE). Double-stranded oligonucleotide of *G/S* linker was prepared by annealing (heating at 95 °C for 5 min and chilling at room temperature for 60 min) of single-strand oligonucleotides, a forward oligonucleotide (5'-TGGAGGAGGAGGATCTGGAGGAGGAGGATCTGGAGGATACCCATACGACTCCAGACTACGCTAT-3', *PacI* site is underlined) and a reverse oligonucleotide (5'-ACCGTACTCTGGGACGTCGTATGGGTATCTCTCCAGATCTCTCTCTCCAGATCTCTCTCTCCAAT-3', *PacI* site is underlined). The resulting oligonucleotides were subcloned into *PacI*-digested pET-DTA-C-CPE, and the sequence was confirmed (pET-DTA-linker-C-CPE).

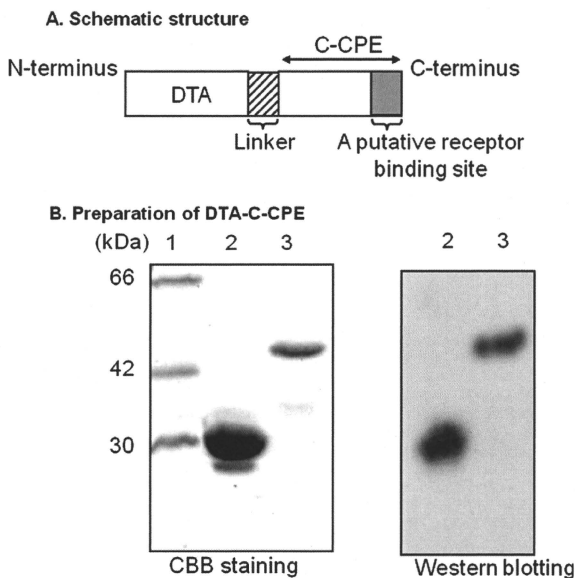


Fig. 1. Preparation of DTA-C-CPE. (A) Schematic structure of DTA-C-CPE. DTA-C-CPE is a fusion protein of DTA and C-CPE with a linker indicated by a slashed column. A dark column indicates a putative receptor-binding region of C-CPE. (B) Preparation of DTA-C-CPE. DTA or DTA-C-CPE was produced by a conventional expression system of *E. coli*, and the proteins were purified by His-tag affinity chromatography with Ni-resins. The purification of DTA-C-CPE was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB) (left panel in B) and by Western blotting using an anti-His-tag mAb (right panel in B). Lane 1, a marker of molecular size; lane 2, DTA; lane 3, DTA-C-CPE. The putative molecular sizes of DTA and DTA-C-CPE were 30 and 43.2 kDa, respectively.

The plasmid, pET-DTA-linker-C-CPE, was transduced into *Escherichia coli* strain BL21 (DE3), after which the cells were cultured in LB medium supplemented with 100 µg/ml ampicillin at 37 °C until the logarithmic phase. Isopropyl- β -thiogalactopyranoside (0.25 mM) was added to the medium, and the cells were cultured for an additional 3 h. The cells were harvested and then lysed in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol). The lysates were centrifuged, and the resultant supernatant was applied to HiTrap Chelating HP (GE Healthcare, Little Chalfont, UK). DTA-C-CPE was eluted by buffer A containing imidazole. The solvent was exchanged with phosphate-buffered saline by using a PD-10 column (GE Healthcare), and the purified protein was stored at -80 °C until use. Purification of DTA-C-CPE was confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue and immunoblotting with anti-His-tag antibody. Protein was quantified by using a protein assay kit (Pierce Chemical, Rockford, IL) with BSA as a standard.

2.4. Cytotoxic activity

Cell viability was determined by using a tetrazolium-based colorimetric assay or lactate dehydrogenase (LDH) assay. Briefly, cells were seeded into a 96-well plate at 1×10^4 cells per well. On the following day, the cells were treated with DTA or DTA-C-CPE (0–20 µg/ml) for 48 h. In the colorimetric assay, WST-8 was added to the wells, mixed thoroughly and incubated for 1 h. Then, the absorbance was measured at 450 nm. In the LDH assay, the release of LDH from the cells was analyzed by using a CytoTox96 NonRadioactive Cytotoxicity Assay kit (Promega, Madison, WI), according to the manufacturer's protocol. The LDH release was calculated by using the following equation: percentage of maximal LDH release = LDH in the culture medium/total LDH in the culture dish.

2.5. Competition assay

Cells (1×10^4 cells) were pretreated with 0–40 µg/ml C-CPE or BSA for 2 h, and then 1 µg/ml of DTA-C-CPE was added. After an additional 48 h of culture, a colorimetric assay was performed as described previously.

3. Results

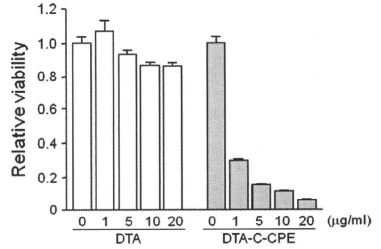
3.1. Preparation of DTA-C-CPE

When DTA enters the cytosol, it inhibits elongation factor 2 through ADP-ribosylation and induces the inhibition of protein synthesis, leading to cell death [20,22]. C-CPE is a receptor-binding domain of CPE, and the CL4-binding region is located on the C-terminal of C-CPE [23]. To prepare a CL4-targeting molecule, we genetically fused DTA with C-CPE at the N-terminal of C-CPE and C-terminal of DTA. A schematic illustration of DTA-C-CPE is shown in Fig. 1A. DTA-C-CPE was produced in *E. coli* and was purified by affinity chromatography with Ni-resins. The molecular size of DTA-C-CPE, as determined by SDS-PAGE and immunoblotting, was identical to its putative size (43.2 kDa, Fig. 1B).

3.2. Cytotoxic properties of DTA-C-CPE

To examine the cytotoxicity of DTA-C-CPE, we investigated the effects of DTA-C-CPE on CL4-expressing L (CL4/L) cells. DTA had no effect on CL4/L cells at 20 µg/ml, whereas DTA-C-CPE dose-dependently decreased the viability, reaching 39.7% relative

A. WST-8 assay



B. LDH release assay

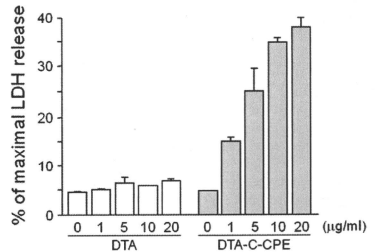


Fig. 2. Cytotoxicity of DTA-C-CPE. CL4/L cells were treated with DTA or DTA-C-CPE at the indicated concentration for 48 h. The cellular viability was measured by WST-8 assay (A) or LDH-release assay (B). Data are the mean \pm SD (n = 3). The data are representative of three independent experiments.

viability at 1 µg/ml (Fig. 2A). Similar results were observed in the LDH-release assay. As shown in Fig. 2B, 5 µg/ml of DTA did not cause a release of cellular LDH; but, DTA-C-CPE at 5 µg/ml significantly increased the release of cellular LDH from 4.7% to 25.0%.

3.3. Targeting properties of DTA-C-CPE

To confirm the CL specificity of DTA-C-CPE, we evaluated the cytotoxicity of DTA-C-CPE in L cells that expressed CL-1, -2, -4 or -5. DAT-C-CPE did not show severe cytotoxicity in L, CL1/L, CL2/L and CL5/L cells, even at 5 µg/ml, whereas DTA-C-CPE reduced the viability of CL4/L cells to 35.0% and 23.3% of the vehicle-treated cells at 1 and 5 µg/ml, respectively (Fig. 3A). To determine whether DTA-C-CPE bound to CL4/L cells via its C-CPE domain, we performed a competition assay. As shown in Fig. 3B, pretreatment of the cells with C-CPE dose-dependently attenuated the cytotoxic activity of DTA-C-CPE from 41.3% to 90.9% of viability at 0–40 µg/ml of C-CPE. In contrast, pretreatment of the cells with BSA at 40 µg/ml did not affect the cytotoxicity of DTA-C-CPE, indicating that DTA-C-CPE bound to the cells via its C-CPE domain. Thus, fusion of C-CPE gives a CL4-targeting property to DTA, producing a CL4-specific cytotoxic agent.

4. Discussion

CL4 is often overexpressed in some malignant tumors, such as breast, prostate, ovarian, pancreatic and gastric cancers [12,14,17]. CL4 targeting is a promising method for tumor-targeting therapy. In the present study, we prepared a fusion protein of DTA, a protein

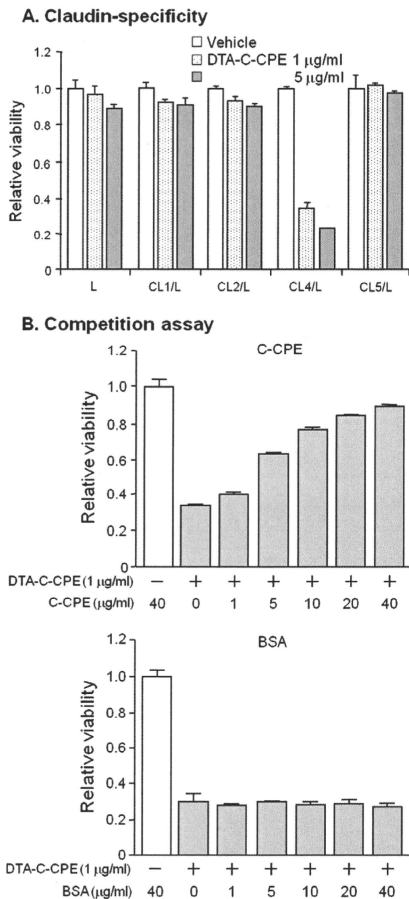


Fig. 3. Cytotoxic properties of DTA-C-PE. (A) Claudin-specificity. L, CL1/L, CL2/L, CL4/L or CL5/L cells were treated with DTA-C-PE at the indicated concentration for 48 h. After incubation, the cellular viability was measured by WST-8 assay. Data are the mean \pm SD ($n=3$). The data are representative of three independent experiments. (B) Competition assay. CL-4/L cells were pretreated with C-PE (upper panel) or BSA (lower panel) at the indicated concentration for 2 h, and then the cells were treated with DTA-C-PE (1 µg/ml) for 48 h. The cellular viability was measured by WST-8 assay. Data are the mean \pm SD ($n=3$). The data are representative of three independent experiments.

synthesis inhibitory factor, and C-PE, which binds to CL-4, and we found that the fused protein (DTA-C-PE) is toxic to CL-4-expressing cells.

DTA kills cells by inactivating elongation factor 2 when one molecule of this protein is introduced into the cytosol [24]. DTA permits the successful targeting of cells displaying only a limited number of tumor-specific growth factor receptors or antigens overexpressed on their surface, and immunotoxins containing

DTA, ONTAK and DT 388GMSCF are used clinically for cancer-targeted therapy [25–27]. Therefore, we selected DTA as a cytotoxic molecule for the present study.

A CL-4-targeting molecule containing DTA needs to bind to CL-4 and enter the cytosol. C-PE is the receptor-binding domain of CPE, and the CL-4-binding region is located on the C-terminal of C-PE [15,23,28]. CL-4 has a sorting signal to clathrin-coated vesicles, and CL-4 is expected to be taken up by clathrin-mediated endocytosis [29–31]. CL-4 bound to DTA-C-PE may be taken up by the endocytotic pathway, followed by release of DTA from endosomes into the cytosol. Further studies are needed to elucidate the detailed mechanism of DTA-C-PE-induced cell death.

Reduced side effects and increased anti-tumor effects are pivotal characteristics needed for anti-tumor agents. Targeting cancer cells by using ligands for growth factor receptors or antigens that are overexpressed on the cell membrane is a potent strategy, and the success of the targeted therapy depends on the target molecule selection. The CL family has attractive characteristics for their use as targets in tumor therapy. First, CL has two extracellular loop domains that can be target sites [12]. Second, CLs are overexpressed in nine of 12 cancer types, creating a differential expression profile between tumor cells and normal cells [12,14]. Third, CLs are often exposed on the apical membrane in cancer cells, whereas CLs are located in the intercellular junction between adjacent cells in normal cells [14]. Even if the CL level in tumors is not more than the level in normal tissues, CL may be more accessible in the tumor. Thus, CLs have great promise as targets for tumor therapy. C-PE is a CL ligand. We prepared C-PE-PSIF, a lead compound for tumor therapy, by using the CL-4-targeting ligand C-PE [32]. We already determined the functional domains of C-PE as a CL-4-targeting molecule, and we are using C-PE as a prototype to develop a novel CL ligand. This is the first study to produce CL-4-targeted DTA. Future development of the CL-4-targeting immunotoxin using DTA and a CL ligand will provide a novel tumor-targeted therapy.

Acknowledgements

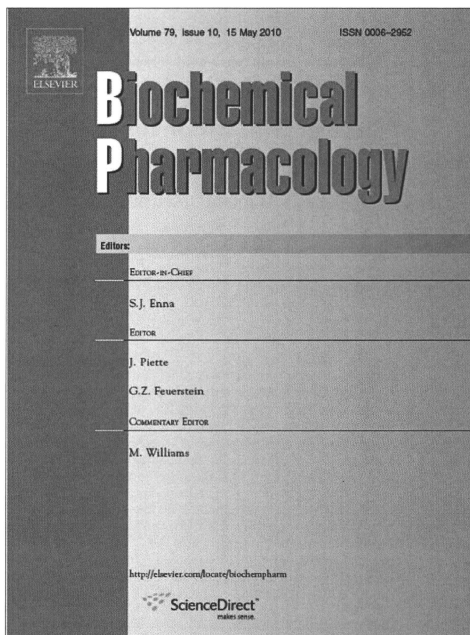
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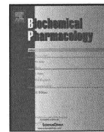
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A claudin-4 modulator enhances the mucosal absorption of a biologically active peptide

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ABSTRACT

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

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1. Introduction

Recent progress in genomic and proteomic technologies has yielded some new biologics, such as peptides, proteins and nucleic

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

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

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

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

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

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

2. Materials and methods

2.1. Materials

Human parathyroid hormone derivative (hPTH(1-34)) was prepared as described previously [23]. n-Dodecyl- β -D-maltoside (DDM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Anti-claudin antibodies and anti-his-tag antibody were obtained from Invitrogen (Carlsbad, CA) and EMD Chemicals Inc. (Darmstadt, Germany), respectively. CMS5 sensor chips, amine-coupling reagents (N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), and ethanolamine-HCl) and HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P20) were obtained from GE Healthcare (Buckinghamshire, UK). All reagents used were of research grade.

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

We prepared expression vectors of N-terminal region-truncated C-CPE184 (amino acids 184–319). These vectors expressed amino acids 194–319 (C-CPE194), 205–319 (C-CPE205), 212–319 (C-CPE212), 219–319 (C-CPE219) and 224–319 (C-CPE224). Insert fragments of each C-CPE mutant were amplified from C-CPE184 cDNA (kindly provided by Dr. Y. Horiguchi, Osaka University) by polymerase chain reaction with forward primers (5'-ATGCTCGAG-ATATAGAAAAGAACTCTT-3' for C-CPE194, 5'-ATGCTCGAGG-TACAGAAGATTAAATTTAACTG-3' for C-CPE205, 5'-ATGGGG-GAGGCTACAGAAAGATTAAATTTAACTG-3' for C-CPE212, 5'-ATGCTCGAGGCTAAGCCCAAGATTAATTTAACTG-3' for C-CPE219, 5'-ATGCTCGATCTATGAAGATTAATTTAACTG-3' for C-CPE224) and a common reverse primer (5'-TTTCTAGCTAAGATTTCTA-

TATTTTTGTCC-3'). The resultant C-CPE fragments were cloned into the pET16b vector. The plasmids were transduced into *E. coli* BL21 (DE3), and protein expression was stimulated by the addition of isopropyl-1-thio- β -D-galactoside. The cell lysates were applied to HisTrapTM HP (GE Healthcare), and C-CPEs were eluted with imidazole. The solvent was exchanged with phosphate-buffered saline (PBS) by gel filtration, and the purified proteins were stored at -80 °C until use. Purification of the proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue. C-CPEs were quantified by using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL) with bovine serum albumin as a standard.

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

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

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

The claudin-displaying BV were diluted with TBS and adsorbed to the wells of 96-well immunoplates (Nunc, Roskilde, Denmark) overnight at 4 °C. The wells were washed with PBS and blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) for 2 h at room temperature. C-CPE or C-CPE derivatives were added to the wells and incubated for an additional 2 h at room temperature. The wells were washed with PBS and incubated with anti-his-tag antibody for 2 h at room temperature. The immuno-reactive proteins were detected by a horseradish peroxidase-labeled secondary antibody with 3,3',5,5'-tetramethylbenzidine as a substrate. The reaction was terminated by the addition of 0.5 M H₂SO₄, and the immuno-reactive proteins were measured at 450 nm.

2.5. Preparation of recombinant claudin-4 protein

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

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

2.6. Surface plasmon resonance (SPR) analysis

SPR measurements were performed with a Biacore T100 instrument (GE Healthcare). Amine-coupling chemistry was used to immobilize claudin-4 at 25 °C on a CMS sensor chip surface docked in a Biacore T100 and equilibrated with HBS-EP+. The carboxymethyl surface of the CMS chip was activated for 2 min with a 1:1 ratio of 0.4 M EDC and 0.1 M NHS at a flow rate of 10 μ l/min. Claudin-4 was diluted to 2.5 μ g/ml in 10 mM MES buffer (pH 6.5) and injected for 2 min over the surface at a flow rate of 10 μ l/min. Excess activated groups were blocked by a 5-min injection of 1 M ethanolamine (pH 8.5) at a flow rate of 10 μ l/min. Approximately 1000 RU of claudin-4 was immobilized by using this protocol. Single-cycle kinetics experiments were performed at 25 °C with a flow rate of 30 μ l/min [27]. C-CPE or its derivatives were serially diluted (1.25, 2.5, 5, 10 and 20 nM) in running buffer (HBS-EP+). Within a single binding cycle, samples of C-CPE or its derivatives were injected sequentially in order of increasing concentration over both the ligand and the reference surfaces. The reference surface, an unmodified flowcell, was used to correct for systematic noise and instrumental drift. Also, prior to the binding cycle for C-CPE or its derivatives, buffer was injected. These "blank" responses were used as a double-reference for the binding data [28]. The sensorgrams were globally fitted by using a 1:1 binding model to determine k_a , k_d and K_D values with the Biacore T100 Evaluation Software version 2.0.1.

2.7. Transepithelial electric resistance (TEER) assay

Caco-2 cells were seeded onto BD BioCoat™ Fibrillar Collagen Cell culture inserts (BD Biosciences, San Jose, CA) at a density of 1×10^5 cells/insert and cultured for 5 days. TJ barriers were formed by a 3-day culture in Entero-STIM™ (BD Biosciences) medium for cellular differentiation. C-CPE or its derivatives were added to the apical side of the chamber. After 18 h of incubation, the TEER values were measured with a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). The percentage changes of TEER values were calculated by the ratio to TEER value in 100 μ g/ml of C-CPE184. EC50 values, at which the TEER ratio is 50%, were calculated by using the four-parameter logistic function of DeltaSoft version 3 (BioMetallics, Princeton, NJ) from dose-response curves of the TEER ratio.

2.8. In situ loop assay

Jejunal absorption of hPTH(1-34) or fluorescein isothiocyanate-dextran with a molecular mass of 4 kDa (FD-4) was evaluated by using an in situ loop assay as described previously [21]. The experiments were performed according to the guidelines of the ethics committee of Osaka University or Asubio Pharma Co. Ltd. After 7-week-old Wister male rats were anesthetized with pentobarbital, a midline abdominal incision was made, and the jejunum was washed with PBS. A 5-cm long jejunal loop was prepared by closing both ends with sutures. hPTH(1-34) (100 μ g) was co-administered with C-CPEs into the loop or administered 4 h after the administration of C-CPEs. Blood was collected from the femoral artery by using a cannulated polyethylene tube at the indicated time points. EDTA (1 mg/ml) was immediately added to the blood sample, and the plasma was recovered by centrifugation. To avoid degradation of hPTH(1-34), aprotinin (500 IU/ml) was immediately added to the plasma, and the plasma was stored at -80 °C until use. The plasma hPTH(1-34) was quantified by

radioimmunoassay (RIA) with anti-hPTH antibody. Anti-hPTH antibody was added to the plasma and then incubated with [¹²⁵I]-Tyr34] hPTH(1-34) (15,000–20,000 cpm/100 μ l) for 24 h. The anti-rabbit IgG goat antibody was added, and anti-hPTH antibody bound to the anti-rabbit IgG goat antibody was separated by centrifugation. The radioactivity in the sediment was counted with a gamma counter (PerkinElmer Inc., Waltham, MA). The area under the plasma concentration time curve (AUC) from 0 to 120 min after administration was calculated by the trapezoidal method. Relative bioavailability (BA) was calculated with the following equation: BA (%) = (AUC (ng·min/ml)/dose (μ g/kg))/(AUC (iv)(ng·min/ml)/dose (iv) (μ g/kg)). AUC (iv) indicates the AUC_{0–120 min} of intravenously administered hPTH(1-34) (10 μ g/kg), and the AUC value is 208.6 ± 52.7 ng·min/ml.

Rats were anesthetized with thiamylal sodium, and a jejunal loop was made, as described above. A mixture of FD-4 (2 mg) and C-CPEs was co-administered into the jejunal loop. Blood was collected from the jugular vein at the indicated time points. The plasma levels of FD-4 were measured with a fluorescence spectrophotometer (Fluoroscan Ascent FL; ThermoElectron Corporation, Waltham, MA). The AUC of FD-4 from 0 to 6 h (AUC_{0–6 h}) was calculated by the trapezoidal method.

2.9. Nasal and pulmonary absorption assay

Nasal and pulmonary absorption of hPTH(1-34) was examined in 7-week-old Sprague–Dawley male rats. The experiments were performed according to the guidelines of the ethics committee of Asubio Pharma Co. Ltd. For the nasal absorption assay, 200 μ g of hPTH(1-34) was intranasally administered to both sides of the nasal cavity 0 or 4 h after nasal administration of C-CPEs. The total injection volume did not exceed 20 μ l. For the pulmonary absorption assay, a polyethylene tube (PE-240, Clay Adams, Becton Dickinson & Co., Sparks, MD) was inserted into the trachea of each rat. A MicroSprayer (Penn-Century, Inc., Philadelphia, PA) was used to perform pulmonary injections of C-CPEs; then, after 0 or 4 h, 150 μ g of hPTH(1-34) was administered with the MicroSprayer. Blood was collected at the indicated time points, and the plasma concentration of hPTH(1-34) was measured by RIA, as described above. AUC and BA (%) values were calculated, as described above.

2.10. Statistical analysis

Data were analyzed by using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, and statistical significance was assigned at $p < 0.05$.

3. Results

3.1. Effects of C-CPE on jejunal, nasal and pulmonary absorption of a peptide drug

We previously found that a claudin-4 modulator, C-CPE184, is a novel type of absorption enhancer by using dextran as a model drug [21]. In the present study, we investigated whether the claudin-4 modulator enhances jejunal, nasal and pulmonary absorption of a peptide drug, hPTH(1-34). When hPTH(1-34) was administered with C-CPE184, C-CPE184 enhanced nasal absorption of hPTH(1-34) by 2.5-fold as compared to the vehicle-treated group. However, C-CPE184 did not enhance jejunal and pulmonary absorption of hPTH(1-34) (Fig. 1 and Table 1). Next, we examined whether pre-treatment of mucosa with C-CPE184 enhanced absorption of hPTH(1-34). When hPTH(1-34) was administered after 4 h of treatment with C-CPE184, the jejunal, nasal and pulmonary absorption of hPTH(1-34) was significantly

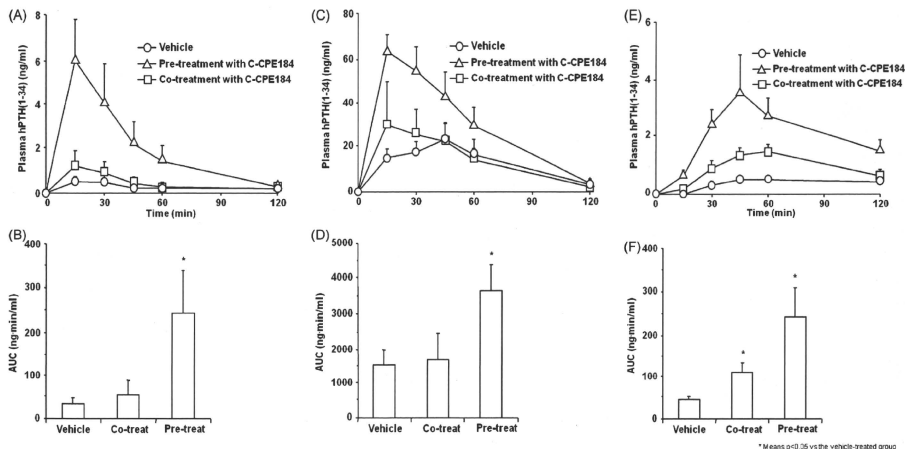


Fig. 1. Effect of C-CPE184 on mucosal absorption of hPTH(1-34) in rats. (A, B) Jejunal absorption of hPTH(1-34). Jejunum was co-treated with hPTH(1-34) (100 µg) and C-CPE184 (20 µg), or jejunum was treated with hPTH(1-34) 4 h after treatment with C-CPE184. (C, D) Pulmonary absorption of hPTH(1-34). hPTH(1-34) (150 µg) was pulmonary administered with C-CPE184 (5 µg) or 4 h after administration of C-CPE184. (E, F) Nasal absorption of hPTH(1-34). hPTH(1-34) (200 µg) was nasally administered with C-CPE184 (2 µg) or 4 h after administration of C-CPE184. Plasma hPTH(1-34) levels were measured at the indicated periods. Time-course changes in plasma hPTH(1-34) levels (A, C, E) and AUC from 0 to 120 min (B, D, F) were calculated. Data are mean ± SE (n = 3–6). Co-treat indicates co-treatment with both hPTH and C-CPE184, and Pre-treat indicates treatment with hPTH 4 h after C-CPE184-treatment. *Significantly different from the vehicle-treated group (p < 0.05).

Table 1
Parameters of mucosal absorption of hPTH(1-34) in C-CPE184-treated rats.

Treatments	Jejunum		Nasal		Pulmonary	
	Cmax (ng/ml)	BA (%) ^a	Cmax (ng/ml)	BA (%)	Cmax (ng/ml)	BA (%)
Vehicle	0.9 ± 0.3	0.4 ± 0.2	0.7 ± 0.1	0.3 ± 0.0	27.8 ± 6.5	14.6 ± 4.4
Co-treat	1.2 ± 0.6	0.6 ± 0.4	1.5 ± 0.2 [*]	0.8 ± 0.2 [*]	35.2 ± 18.9	14.9 ± 6.5
Pre-treat	6.0 ± 1.8 [*]	2.7 ± 0.9 [*]	3.6 ± 1.3	1.4 ± 0.4 [*]	67.6 ± 7.5 [*]	34.5 ± 6.6 [*]

^a BA (%) = (AUC/Dose)/(AUC iv/Dose iv).

Data are means ± SE.

^{*}p < 0.05, [†]p < 0.01, as compared to vehicle-treated group.

increased 7.5-, 5.6- and 2.4-fold compared to the vehicle-treated group (Fig. 1 and Table 1).

3.2. Preparation of N-terminal-truncated C-CPE184-319 derivatives

The solubility of C-CPE184 is less than 0.3 mg/ml in PBS due to its hydrophobicity (Table 2). An increase in solubility without loss of claudin-4-modulating activity might improve the mucosal-absorption-enhancing activity of C-CPE184. Van Itallie et al. showed that the removal of the 10 N-terminal amino acids from C-CPE184 to yield C-CPE194 results in high solubility (10 mg/ml)

Table 2
Solubility of C-CPE184 and the N-terminal-truncated mutants.

Derivatives	Molecular size (kDa)	Solubility ^a (mg/ml)
C-CPE184	18.2	<0.3
C-CPE194	17.3	>10
C-CPE205	16.1	>4
C-CPE212	15.4	Insol. ^b
C-CPE219	14.7	Insol.
C-CPE224	14.2	Insol.

^a Solvent is PBS.

^b Insol., insoluble.

[26]. Although C-CPE194 is a claudin-4 binder, whether C-CPE194 modulates the TJ-barrier remains unclear. C-CPE194 contains nine β-sheets and one α-helix, and its 16 C-terminal amino acids are believed to comprise the claudin-4-binding region (Fig. 2A) [26,29]. Based on this information, we prepared five different N-terminal-truncated C-CPE184 derivatives: C-CPE194, which lacks the 10 N-terminal amino acids; C-CPE205, which is truncated prior to the β1-sheet; C-CPE212, which is truncated after the β1-sheet; C-CPE219, which is truncated after the α-helix; and C-CPE224, which is truncated before the β2-sheet (Fig. 2A). The C-CPes were expressed in *E. coli* (Fig. 2B). The solubility of C-CPE194 (>10 mg/ml) and C-CPE205 (>4 mg/ml) in PBS was greater than that of C-CPE184 (<0.3 mg/ml) (Table 2). However, C-CPE212, C-CPE219 and C-CPE224 formed solid inclusion bodies in *E. coli*, and these inclusion bodies could not be dissolved without 2 M urea. Therefore, further experiments were performed with C-CPE184, C-CPE194 and C-CPE205.

3.3. Characterization of C-CPE194 and C-CPE205

To study the interaction between the C-CPes and claudin-4, we performed ELISA with claudin-4-displaying BV, as described previously [24]. When C-CPes were added to claudin-4-displaying

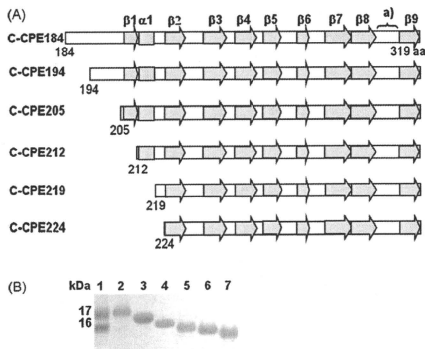


Fig. 2. Preparation of C-CPEs. (A) Schematic structure of C-CPEs. Van Itallie et al. determined the 3-dimensional structure of C-CPE194 containing nine β -sheets and one α -helix [26]. Based on the structural information, we designed five N-terminal truncated C-CPE184 derivatives. (B) CBB staining. C-CPEs were prepared and then purified by affinity chromatography. Lane 1, a maker for molecular weight; lane 2, C-CPE184; lane 3, C-CPE194; lane 4, C-CPE205; lane 5, C-CPE212; lane 6, C-CPE219; lane 7, C-CPE224.

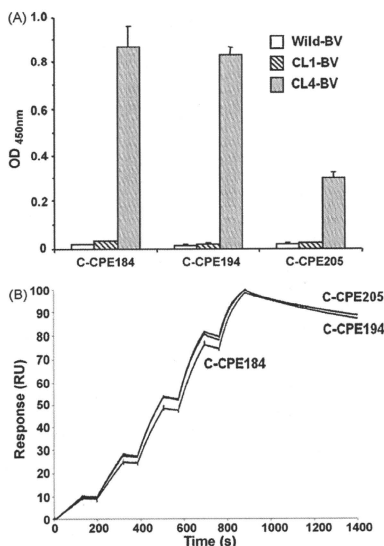


Fig. 3. Interaction of C-CPEs with claudin. (A) ELISA. The immunoplate was coated with wild-type BV (Wild-BV), claudin-1-displaying BV (CL1-BV) or claudin-4-displaying BV (CL4-BV), and then C-CPEs were added to the well. C-CPEs bound to BVs were detected by the addition of anti-his tag antibody and a labeled secondary antibody. Data are means \pm SD ($n = 3$). (B) SPR assay. Claudin-4 was immobilized on a CMS sensor chip by the amine-coupling method. C-CPEs were injected sequentially at concentrations of 1.25, 2.5, 5, 10 and 20 nM. The association phase was monitored for 120 s at a flow rate of 10 μ l/min, and the dissociation phase was followed for 600 s at the same flow rate. The maximum values of response (R_{max}) for all curves were compensated to 100 RU.

Table 3
Binding kinetics of C-CPEs to claudin-4.

Derivatives	k_a (1/Ms)	k_d (1/s)	K_D
C-CPE184	5.96×10^5	2.55×10^{-4}	429 pM
C-CPE194	7.13×10^5	3.24×10^{-4}	455 pM
C-CPE205	7.67×10^5	2.87×10^{-4}	374 pM

BV-adsorbed immunoplates, C-CPE194 and C-CPE205 bound to claudin-4-displaying BV but not mock BV or claudin-1-displaying BV (Fig. 3A). We performed SPR analysis to compare the affinities of C-CPEs to claudin-4. Claudin-4 proteins were fixed on the sensor chip, and C-CPEs were injected. Then, we measured the interaction between claudin-4 and C-CPEs. As shown in Fig. 3B and Table 3, C-CPE184, C-CPE194 and C-CPE205 had almost the same affinity to claudin-4 with K_D values of 429, 455 and 374 pM, respectively. The association and dissociation rates of C-CPE194 and C-CPE205 were also similar to those of C-CPE184. C-CPE184, C-CPE194 and C-CPE205 showed similar TJ-modulating activities in Caco-2 monolayer cells; their EC50 values were 0.49, 0.57 and 0.51 μ g/ml, respectively (Fig. 4A and Table 4). We performed in situ loop assays to examine the jejunal absorption of FD-4 by C-CPEs. C-CPE194 and C-CPE205 enhanced the jejunal absorption of FD-4 similar to C-CPE184 at 0.2 mg/ml (Fig. 4B–D). Treatment with C-CPE194 or C-CPE205 at 1.0 mg/ml yielded a greater and earlier absorption of FD-4 than treatment at 0.2 mg/ml (Fig. 4B, C). We could not test 1.0 mg/ml of C-CPE184 due to its low solubility.

3.4. Jejunal and pulmonary absorption of hPTH(1–34) by co-treatment with C-CPE194

C-CPE194 enhanced the jejunal absorption of FD-4 to a similar extent as C-CPE184 and C-CPE205; C-CPE194 was also 30- and 3-fold more soluble than C-CPE184 and C-CPE205, respectively. C-CPE194 enhanced the jejunal absorption of hPTH(1–34) at 0.2 and 4.0 mg/ml (Fig. 5A). The AUC values were increased 11.0- and 18.4-fold as compared to the vehicle-treated group (Fig. 5B), and the C_{max} and BA of the jejunal absorption of hPTH(1–34) were also increased by C-CPE194 (Table 5). Additionally, the pulmonary absorption of hPTH(1–34) was enhanced by C-CPE194 (AUC = 3080.0 ± 1994.3 ng·min/ml in vehicle-treated group, AUC = $13,397.7 \pm 5830.1$ ng·min/ml in C-CPE194 (0.8 mg/ml)-treated group) (Fig. 5C, D). The C_{max} and BA of hPTH(1–34) were also increased by C-CPE194 (Table 5).

4. Discussion

Biologics are generally hydrophilic and poorly absorbed by the mucosa; therefore, many biologics are administered via injection. The development of a delivery system to allow biologics to pass across the epithelial barrier in mucosa is a pivotal issue for pharmaceutical therapy with biologics, since mucosal administration is needle-free, non-invasive, convenient and comfortable for patients [30,31]. We previously found that C-CPE184 enhanced jejunal absorption of dextran with a molecular mass of <10 kDa through its modulation of the claudin-4 barrier [21]. In the present study, we investigated the effect of a claudin-4 modulator on the mucosal absorption of a biologic, hPTH(1–34), and we found that a claudin-4 modulator is also a potent jejunal, nasal and pulmonary absorption enhancer of this biologic.

CPE is a 35-kDa polypeptide consisting of 319 amino acids [32]. The functional domain of CPE is divided into an N-terminal toxic domain and a C-terminal receptor-binding domain [33]. The receptor-binding fragments of CPE correspond to amino acids 169–319, 171–319, 184–319, 194–319 and 290–319 [20,26,33–35]. Among these fragments, only C-CPE184 and C-CPE194 have been

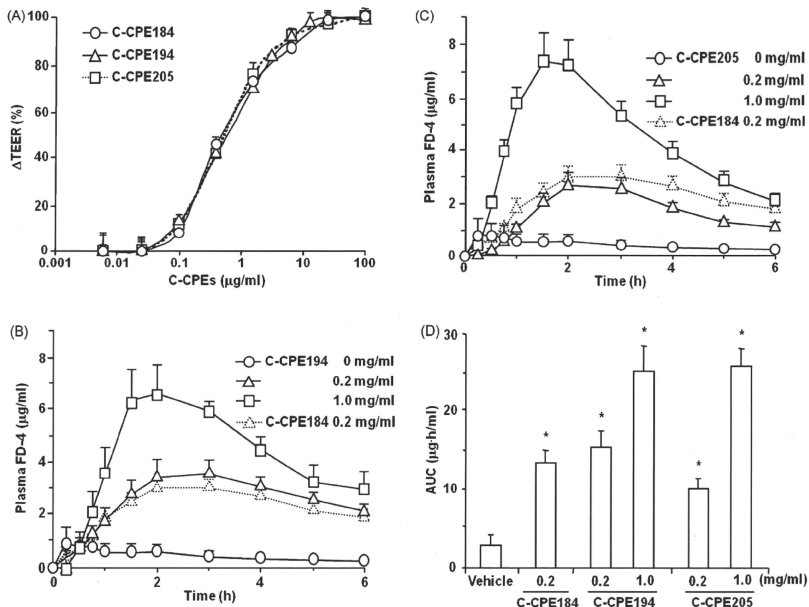


Fig. 4. Modulation of the TJ-barrier by C-CPEs. (A) Caco-2 cells were seeded on a BioCoat™. When TJ-barriers were developed and the cell sheets reached a plateau in their TEER value, C-CPEs were added to the wells from the basal side at the indicated concentration. After 18 h, the TEER was measured. The Δ TEER was calculated as the ratio to reduced TEER values at 0 and 100 $\mu\text{g/ml}$ of C-CPE184 as 0% and 100%, respectively. Data are mean \pm SD ($n = 3$). (B–D) Jejunal absorption of FD-4. Jejunum were treated with FD-4 and C-CPEs at the indicated concentration. Time-course changes in plasma FD-4 levels (B, C) and AUC from 0 to 6 h (D). Data are means \pm SE ($n = 4–9$). *Significantly different from the vehicle-treated group ($p < 0.05$).

proven to bind to claudin-4 [10,26], and a mucosal-absorption-enhancing effect was proven only for C-CPE184 [21]. The claudin-4 modulator C-CPE184 is a 400-fold more potent jejunal absorption enhancer of dextran as compared to a clinically used absorption enhancer, sodium caprate [21]. However, the low solubility of C-CPE184 (<0.3 mg/ml in PBS) has limited its applicability. This low solubility may result in the slow onset of TJ opening due to limiting the access of C-CPE184 to claudin. Last year, Van Itallie et al. made a breakthrough by truncating the N-terminal of C-CPE184 by 10 amino acids to yield C-CPE194 [26]. They found that C-CPE194 has affinity to claudin-4 and high solubility (>10 mg/ml); moreover, they determined the 3-dimensional structure of C-CPE194, which contains nine β -sheets and one α -helix, and they suggested that the intervening surface loop spanning region 304–312 (located between the $\beta 8$ and $\beta 9$ sheets) may be a claudin-binding domain. Based on the structural data for C-CPE194, we prepared five N-terminal-truncated C-CPE184 derivatives: C-CPE194, C-CPE205, C-

CPE212 (without the $\beta 1$ sheet), C-CPE219 (without the $\beta 1$ sheet and α helix), and C-CPE224 (without the $\beta 1$ sheet and α helix). C-CPEs lacking the $\beta 1$ sheet are soluble in PBS containing 2 M Urea but insoluble in PBS. C-CPE184, C-CPE194 and C-CPE205 have almost the same kinetics parameters for binding to claudin-4 and the same TJ-barrier modulating activity (Table 3, Fig. 4A). Thus, the $\beta 1$ sheet appears to be critical for maintaining the structure of C-CPE, and the N-terminal region corresponding to amino acids 184–204 may not be involved in claudin-4 binding or TJ-barrier modulation.

Biologics must escape degradation by mucosal enzymes to be absorbed by the mucosa. C-CPE184 (0.2 mg/ml) did not enhance jejunal or pulmonary absorption of hPTH(1–34). However, when hPTH(1–34) was administered 4 h after treatment with C-CPE184, jejunal, pulmonary and nasal absorption was enhanced. Thus, hPTH(1–34) may be degraded in the jejunal and pulmonary mucosa before the enhancement of its absorption by co-administered C-CPE184. Indeed, another claudin-4 modulator, C-CPE194, which is 30-fold more soluble than C-CPE184, significantly enhanced the jejunal and pulmonary absorption of hPTH(1–34). These findings indicate that modulation of claudin-4 may be a potent strategy for mucosal-absorption enhancement of biologics.

Meanwhile, a critical issue in the clinical application of the claudin-4 modulator as a mucosal-absorption enhancer is its safety. Problems with the safety of a claudin-4 modulator include the safety of a claudin-4 modulator in itself and the safety of the modulation of claudin-4, i.e., entry of unwanted substances by the

Table 4
TJ-modulating activities of C-CPEs in Caco-2 cells.

Derivatives	EC50 values ^a
C-CPE184	0.49 $\mu\text{g/ml}$
C-CPE194	0.57 $\mu\text{g/ml}$
C-CPE205	0.51 $\mu\text{g/ml}$

^a The concentration of C-CPEs at which a 50% decrease in TEER value was observed in Fig. 4A.

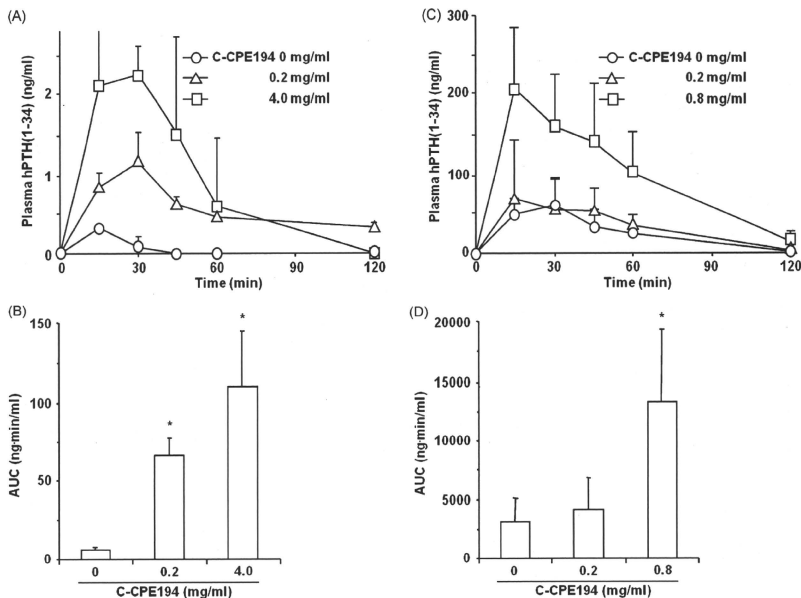


Fig. 5. Mucosal absorption of hPTH(1-34) by a claudin-4 modulator. (A, B) jejunal absorption of hPTH(1-34). Rat jejunum was treated with hPTH(1-34) (100 µg) and C-PE194 at the indicated doses. Time-course changes in plasma hPTH(1-34) (A) and AUC from 0 to 120 min (B) were analyzed. (C, D) Pulmonary absorption of hPTH(1-34). hPTH(1-34) (150 µg) and C-PE194 at the indicated doses were pulmonary administered, and time-course changes in plasma hPTH(1-34) concentration (C) and AUC from 0 to 120 min (D) were analyzed. Data are mean ± SE (n = 3). *Significantly different from the vehicle-treated group (p < 0.05).

opening of TJs. As mentioned above, C-CPEs are the only claudin-4 modulator. C-CPEs are polypeptide fragments of CPE consisting of more than 120 amino acids, and C-CPEs themselves may have antigenicity. Claudin-4 modulator significantly enhanced jejunal absorption of dextran with a molecular mass of less than 10 kDa [21], and C-CPEs (>14.2 kDa) may not be absorbed by the modulation of claudin-4. Moreover, *C. perfringens* are indigenous bacterium, and immunological tolerance may be induced. Thus, the antigenicity of C-CPE might be partly negligible. Because absorption enhancers would be used as an additive in drugs, they would be repeatedly administered. To avoid the risk of antigenicity, the development of a chemical compound-type or a 30–40 mer peptide-type of claudin modulator is needed. The determination of the 3-dimensional structure of claudin is also important for the theoretical development of promising claudin modulators. Ling et al. prepared a 12-mer peptide-type claudin-4 binder which did

not modulate TJs [36], and Van Itallie et al. determined the structure of C-CPE [26]. A peptide-type claudin modulator will be developed in the near future.

The other safety issue is the possible influx of unwanted substances that could be caused by the opening of TJs. C-CPE has demonstrated no damages to mucosal epithelial tissue in rat intestine [21]. Treatment of cells with C-CPE decreased the level of intracellular claudin-4 proteins paralleled by a disruption of the TJ-barrier [10]. Claudin contains the clathrin-sorting signal in its C-terminal intracellular domain, and claudin was often internalized [37,38]. Taken together, these results indicate that C-CPEs may disrupt the TJ-barrier, allowing the movement of solutes through the paracellular route. Do claudin modulators reversibly modulate the TJ-barrier and specifically regulate the movement of solutes? Disruption of the TJ-barrier by C-CPE is reversible, and the TJ-barrier gradually recovered after the removal of C-CPE [10]. The

Table 5
Parameters of mucosal absorption of hPTH(1-34) in C-PE 194-treated rats.

C-PE194 (mg/ml)	Jejunum		C-PE194 (mg/ml)	Pulmonary	
	Cmax (ng/ml)	BA (%) ^a		Cmax (ng/ml)	BA (%)
0	0.3 ± 0.0	0.1 ± 0.0	0	62.3 ± 32.8	26.5 ± 17.4
0.2	1.2 ± 0.4 [*]	0.8 ± 0.2 ^{**}	0.2	78.7 ± 66.1	34.1 ± 21.6
4.0	2.8 ± 0.2 [*]	1.3 ± 0.3 ^{**}	0.8	205.2 ± 79.4 [*]	100.6 ± 39.3 [*]

^a BA (%) = (AUC/Dose)/(AUC iv/Dose iv).

Data are mean ± SE.

^{*}p < 0.05, ^{**}p < 0.01 as compared to the vehicle-treated group.

quick recovery of TJ-barriers will need to be facilitated. One approach is the development of a quickly reversible claudin modulator. Another approach is the development of a claudin inducer for the combination of a claudin modulator and inducer. Another approach is the reduction of unwanted transport using the properties of claudins. Claudin comprises a multigene family consisting of 24 members. Claudin forms paired TJ strands by polymerization in a homomeric and heteromeric manner, and the claudin strands interact in a homotypic and heterotypic manner between adjacent cells [39,40]. TJ-barrier properties are believed to be determined by the combination and mixing ratios of claudin species [41]. Interestingly, the diversity of claudin may contribute to the regulation of specific solute movement through the paracellular route [17]. The expression profiles of claudin in mucosal epithelium exhibit heterogeneity [13–15,42]. The development of claudin modulators with solute and tissue specificity will reduce the non-specific influx of substances caused by the modulation of TJs.

In summary, we found that claudin-4 modulator enhanced the jejunal, pulmonary and nasal absorption of a peptide drug. This report is the first to indicate that a claudin-4 modulator may be a mucosal-absorption enhancer of biologics.

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Prevention of hepatic ischemia–reperfusion injury by pre-administration of catalase-expressing adenovirus vectors

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Hepatectomy

ABSTRACT

Liver ischemia/reperfusion (I/R) injury, which is mainly caused by the generation of reactive oxygen species (ROS) during the reperfusion, remains an important clinical problem associated with liver transplantation and major liver surgery. Therefore, ROS should be detoxified to prevent hepatic I/R-induced injury. Delivery of antioxidant genes into liver is considered to be promising for prevention of hepatic I/R injury; however, therapeutic effects of antioxidant gene transfer to the liver have not been fully examined. The aim of this study was to examine whether adenovirus (Ad) vector-mediated catalase gene transfer in the liver is an effective approach for scavenging ROS and preventing hepatic I/R injury. Intravenous administration of Ad vectors expressing catalase, which is an antioxidant enzyme scavenging H₂O₂, resulted in a significant increase in catalase activity in the liver. Pre-injection of catalase-expressing Ad vectors dramatically prevented I/R-induced elevation in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, and hepatic necrosis. The livers were also protected in the another liver injury model, CCl₄-induced liver injury, by catalase-expressing Ad vectors. Furthermore, the survival rates of mice subjected to both partial hepatectomy and I/R treatment were improved by pre-injection of catalase-expressing Ad vectors. On the other hand, control Ad vectors expressing β-galactosidase did not show any significant preventive effects in the liver on the models of I/R-induced or CCl₄-induced hepatic injury described above. These results indicate that hepatic delivery of the catalase gene by Ad vectors is a promising approach for the prevention of oxidative stress-induced liver injury.

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1. Introduction

Hepatic ischemia/reperfusion (I/R) injury occurs in a variety of clinical settings, such as in liver transplantation, hepatic failure after shock, and liver surgery, and results in severe damages that substantially contribute to the morbidity and mortality of such cases [1–3]. Hepatic I/R injury is caused by reactive oxygen species (ROS), including superoxide anion, hydrogen oxide, and hydroxyl radical, which are generated by reperfusion of the ischemic tissue. ROS induce lipid peroxidation and damages to proteins and nucleic acids, leading to parenchymal cell dysfunction and necrosis, increased vascular

permeability, and inflammatory cell infiltration [4]. Therefore, ROS should be detoxified to prevent hepatic I/R injury.

In previous animal studies, antioxidative enzyme catalase and superoxide dismutase (SOD) were systemically administered to neutralize ROS and prevent I/R-induced hepatic injury. Although catalase and SOD are endogenously expressed in the cells, the expression levels of these enzymes are insufficient to prevent I/R injury. Administration of antioxidant enzymes exhibited therapeutic effects on ROS-induced diseases, including I/R-induced hepatic injury, in several studies [5–8]; however, these enzymes are known to be rapidly eliminated from the circulation following systemic administration, which limits their therapeutic potential, [9,10] although chemical modification of antioxidant enzymes has been carried out to enhance their plasma half-lives and tissue accessibility [5,6,9]. In addition, systemically administered antioxidant enzymes might be degraded in the endosomes/lysosomes because they are internalized into the cells via the endocytosis pathway.

The delivery of therapeutic genes encoding antioxidant enzymes into the liver is considered to be a promising strategy to overcome these problems. Previous studies have demonstrated that ROS-mediated injury was efficiently prevented by over-expression of antioxidant

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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 catalyzes 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 catalyzing 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 pCMV β (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 P1-SceI, and ligated with I-CeuI/P1-SceI-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 (Qagen, Inc., Valencia, CA) according to the manufacturer's instructions. The viruses were prepared by the standard method, then purified with CCl_4 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 transfected 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 μ g), 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 non-specific 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 transfected 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-CLT 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 administered 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 \pm 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,

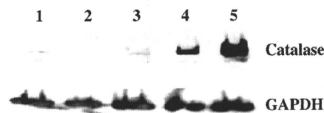


Fig. 1. Catalase expression following Ad vector transduction. A549 cells were transfected with Ad-LacZ, Ad-CAT, or AdK7-CAT at 3000 VP/cell for 2 h. Protein samples were collected after a 48-h incubation and analyzed by Western blotting. Lane 1, mock; lane 2, Ad-LacZ; lane 3, AdK7-LacZ; lane 4, Ad-CAT;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

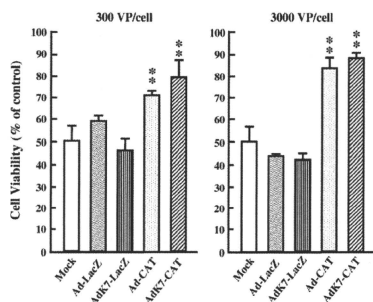


Fig. 2. Protective effects of catalase-expressing Ad vectors against menadione-induced cell death. HepG2 cells were transfected with Ad vectors at 300 or 3000 VP/cell for 2 h. After a 48-h incubation, menadione was added to the medium at a final concentration of 30 mM, and the cells were cultured for 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 \pm 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 CCl₄-induced liver injury

To explore whether Ad vector-mediated catalase expression prevents other types of oxidative stress-induced liver injury, CCl₄ was intraperitoneally injected into mice pretreated with catalase-expressing Ad vectors. CCl₄ is well known to produce CCl₃ radical, leading to acute liver injury. Serum ALT and AST levels were highly elevated following CCl₄ 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 CCl₄-

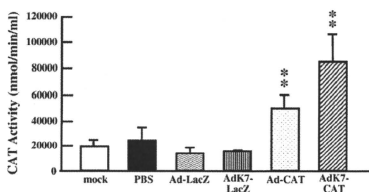


Fig. 3. Catalase activity in the liver following intravenous administration of catalase-expressing Ad vectors. Ad vectors were administered to mice at the dose of 1×10^{10} VP/mouse. The livers were recovered 48 h after injection, and catalase activities in mouse liver homogenates were determined. The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

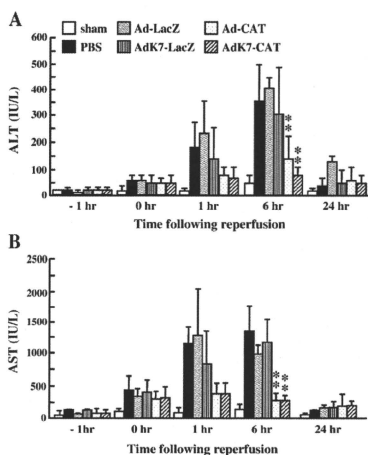


Fig. 4. Effects of pre-administration of catalase-expressing Ad vectors on serum ALT (A) and AST (B) levels in mice following hepatic ischemia/reperfusion injury. Ad vectors were intravenously administered to mice at a dose of 1×10^{10} VP/mice. Forty-eight hours after Ad vector injection, mice were 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 \pm S.E. ($n = 3-8$). *Significantly different from the PBS-injected group at $p < 0.01$.

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.

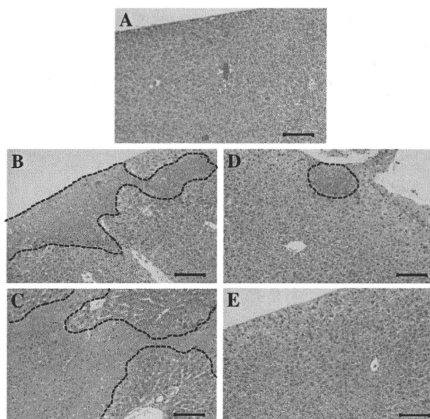


Fig. 5. Representative images of liver sections of mice 24 h following hepatic ischemia/reperfusion. A) Sham, B) PBS, C) AdK7-LacZ, D) Ad-CAT, and E) AdK7-CAT. Mice were subjected to hepatic I/R 48 h after Ad vector injection, as described in Fig. 4. Livers were recovered 24 h after I/R treatment, and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