表1　タイトジャンクション研究

年代	研究内容
1973年	TJストランドの発見
1982年	脂質ミセル説の提唱
1993年	Occludinの発見
1998年	Claudinの発見
1999年以降	Claudinバリア機能の証明
2005年	Tricellulinの発見

表2　既存の生体バリアを利用した創薬研究

吸収促進剤	生体内標的分子
EDTA	Ca^{2+}のキレート
オレイン酸	脂質二重膜の流動性亢進
NO供与剤	不明
カプリン酸ナトリウム	ホスホリパーゼC

免疫組織で高発現していること[11]，occludinおよびclaudinがC型肝炎ウイルスの感染受容体であることも報告されており[12,13]，TJ分子基盤を利用した粘膜ワクチンおよび感染阻害薬創出の可能性も見いだされている．

このように，月田グループによるoccludinおよびclaudinの発見に端を発した上皮細胞バリア研究の進展に伴い，従来までの「上皮細胞バリア＝物質透過障壁」という概念に加え，「上皮細胞バリア分子基盤＝創薬ターゲット」という新たな萌芽が生まれつつある．

本稿では，上述した本邦発の新規創薬ターゲット，上皮細胞バリア制御分子claudinの可能性について，薬物送達法および抗がん剤開発に焦点を絞り，当研究グループの研究成果を概説する．

上皮細胞バリアの分子基盤

隣接する細胞は10～20 nmの空隙を隔てて向かい合っており，アピカル側では隣接する細胞膜がストランド様の構造体TJによって密着し，細胞間の距離がゼロとなるキッシングポイントにより細胞間隙における物質の移動が制御されている[1]．電子顕微鏡を用いた解析から，TJストランドは網目状にラテラル面に広がり物質の移動を制御していることが1970年代には明らかにされていたものの，このストランドの本体については脂質ミセル説なども提唱され長年にわたり議論が続いていた(表1)[14]．

1993年に，京大月田グループにより，はじめてのTJ構成蛋白質としてoccludinが見いだされ，TJストランドが蛋白質で構成されていることが示された[2]．1998年に新たなTJ構成蛋白質としてclaudinが同定され，1999年以降claudinがTJバリア能の本体を担うことを示唆する知見が集積しつつある[3,15,16]．さらに2005年には，同グループにより，上皮細胞層において三つの細胞が交わるジャンクション(トリセルラージャンクション)の構成蛋白質としてtricellulinが同定されている[17]．Tricellulinをノックダウンすると上皮細胞バリア機能が低下することから，上皮細胞バリアは2細胞間のシール機構と3細胞間のシール機構により構成されていることが実験的に証明された[17]．

上皮細胞バリアを利用した第一世代の創薬研究

前述したように，上皮細胞層は生体内外を隔てる障壁として機能しており，薬物吸収に際しては上皮細胞層の透過が不可欠となっている．すでに30年あまりにわたり上皮細胞バリアを利用した創薬研究として吸収促進剤が研究されてきており，EDTA，オレイン酸，NO供与剤，カプリン酸ナトリウムなどが吸収促進活性物質として見いだされている(表2)．それぞれの作用点は細胞間隙に存在するカルシウムイオン，細胞膜，phospholipase Cなどであり，TJの開口を通じて吸収促進効果を発揮すると考えられている．

これらアプローチが研究開発されていた1980年～98年にはTJ構成蛋白質は未解明であり，吸収促進作用に組織特異性が乏しいこと，TJの開口に伴い薬物以外の物質の非特異的な流入が生じることなどから，臨床応用されている吸収促進剤はカプリン酸ナトリウムなどにすぎない[18～20]．

上皮細胞バリアを利用した第二世代の創薬研究

当研究グループでは，上述した吸収促進剤の有する課題はTJを利用した薬物送達法の限界を示しているのではなく，TJの分子基盤に立脚したア

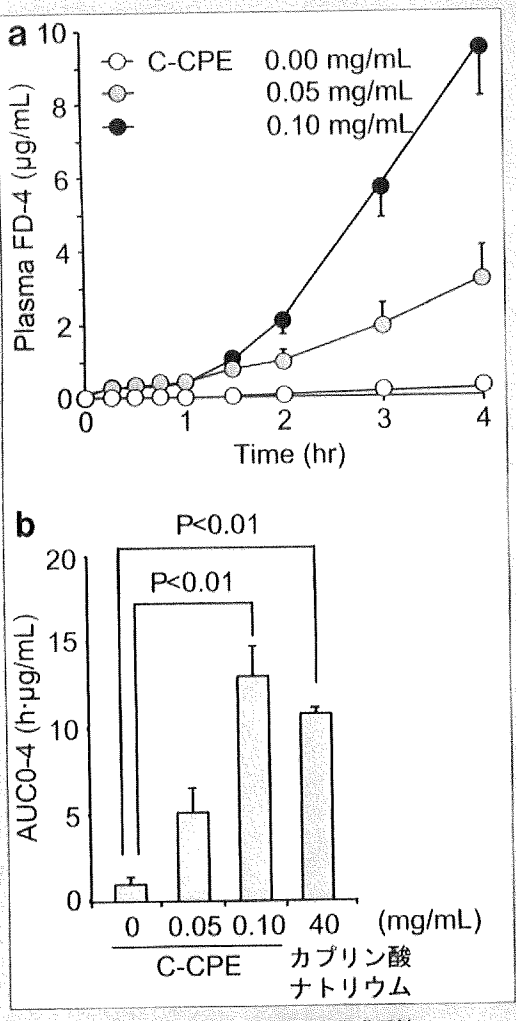

図1 C-CPEの腸管吸収促進活性

a：血漿中FD-4濃度の経時変化，**b**：AUC値
FITCラベルした分子量4000のデキストラン(FD-4)をモデル薬物として用いて，ラット腸管ループ法によりC-CPEの吸収促進活性を解析(Kondoh M et al, 2005[25]を一部改変)．

プローチが採られてこなかったことに起因していると考え，上述した吸収促進剤を第一世代のTJ modulatorと定義し，TJの分子基盤に立脚した第二世代のTJ modulatorを利用した薬物送達研究を進めてきた[21]．

1993年，京大月田グループによりTJ構成蛋白質として4回膜貫通蛋白質occludinが見いだされ，TJが蛋白質によって構成されていることがはじめて明らかにされた[2]．しかしながら，occludinを欠損させても，機能的，構造的に正常なTJが構成されていたことから，occludinはTJバリア機能分子ではないことが示唆された[22]．この報告とほぼ時を同じくして，1998年に新たなTJ構成蛋白質claudinが同定された(**表1**)[3]．

Claudinは分子量23 kDaの4回膜貫通蛋白質であり，現在までに24種類の分子が見いだされている．非常に興味深いことに，発現およびバリア機能には組織特異性が認められ，claudin-1欠損マウスでは重層上皮細胞のバリア機能が異常をきたし，分子量600程度の分子が皮膚を透過し，claudin-5欠損マウスでは血液脳関門を分子量800程度の分子が通過する[5,6,8,15,23]．さらに，claudin-11は血液精巣関門バリアを担っていることも明らかにされている[7]．これらの報告はclaudinを分子特異的に制御することができれば新たな薬物送達法の開発に繋がることを意味している．

現在のところ，claudinのバリア機能を阻害する分子としてはウエルシュ菌エンテロトキシン(CPE)のC末断片(C-CPE)のみがclaudin-4のバリア機能を阻害する分子として知られている[16,24]．そこでC-CPEをclaudin modulatorのモデル分子として用いて，claudinを利用した薬物送達法創出の可能性について検証を試みた．

まず，C-CPEの薬物送達活性を分子量4000のデキストラン(FD-4)をモデル分子として用いてラット腸管ループ法により解析したところ，C-CPE添加濃度依存的に血漿中FD-4濃度は上昇していた(**図1a**)．C-CPEの吸収促進活性は臨床応用されている吸収促進剤であるカプリン酸ナトリウムに比して400倍もの値を示しており(**図1b**)，さらに吸収促進効果には組織特異性も観察されていた[25]．

CPEはC末30アミノ酸を介して受容体と相互作用することが示唆されている[26]．そこで，C-CPEの薬物送達活性におけるclaudin-4の関与を検討するために，受容体結合領域をすべて欠損させたC-CPE289およびその一部を欠損させたC-CPE303を作成し，ラット腸管における吸収促進活性を解析したところ，C-CPE289，C-CPE303処理では吸収促進活性が著しく減弱していた(**図2a, b**)．

以上の結果より，claudinを利用した粘膜吸収促進法の有用性が示唆される．

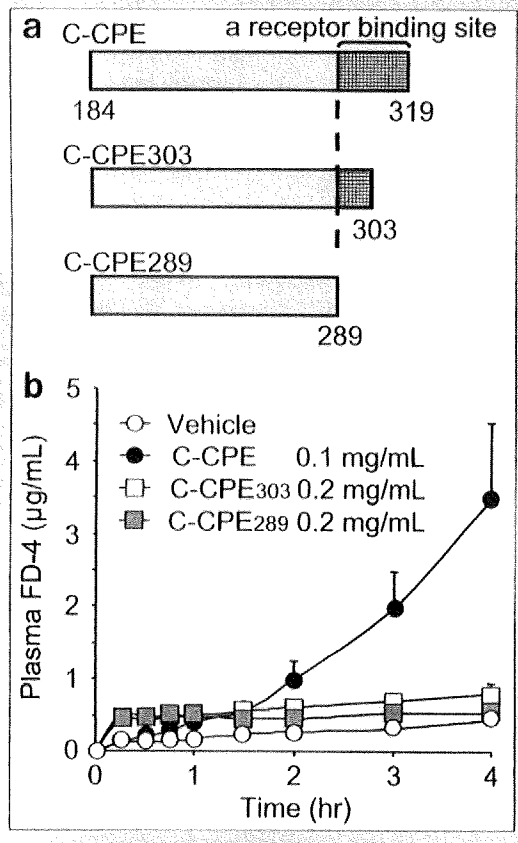

図2　C-CPE の吸収促進作用における claudin-4 の関与

a：C-CPEs，**b**：腸管吸収促進効果

C-CPE の claudin-4 結合領域を欠損させた変異体(**a**)の FD-4 吸収促進活性を解析(**b**)．なお，データは Kondoh M et al., 2005[25]を一部改変．

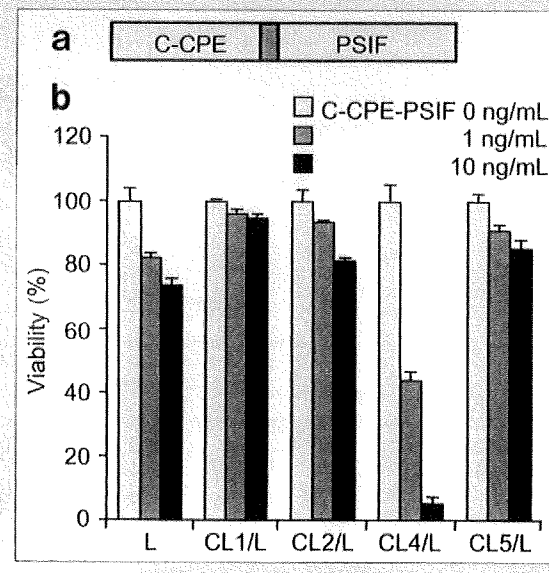

図3　Claudin-4 指向性分子

a：C-CPE-PSIF，**b**：Claudin(CL)特異性

Pseudomonas exotoxin の蛋白質合成阻害ドメイン(PSIF)と C-CPE との融合体(**a**)を作製し，各種 claudin 発現細胞に各濃度 24 時間処理後に WST アッセイにより細胞毒性を解析(**b**)．なお，データは Saeki R et al., in press[28]を一部改変．

上皮細胞バリアを利用した第三世代の創薬研究

上述したように，従来の生体バリアを利用した創薬研究は薬物吸収促進に焦点が当てられてきた．2000 年以降，悪性腫瘍(年間死者数 700 万人)の 90%を占める上皮由来のがんと claudin との関連性についても，多方面からの研究が進み，卵巣がん，膵臓がん，膀胱がんなど 12 種類あまりのがんにおける claudin の高発現が見いだされ，claudin ががんターゲティングの標的分子としても注目されている[9,10]．

さて，正常な上皮細胞層では水平方向に細胞は分裂しコンタクトインヒビッションにより細胞増殖は制御されている．一方，上皮細胞ががん化すると細胞分裂軸が回転し垂直方向への分裂がはじまり，コンタクトインヒビッションがかからず腫瘍組織を形成していくと考えられている．このがん化早期イベントである分裂軸の回転を利用したがん治療戦略を構築することができれば，がんの早期診断・早期治療法の開発に繋がるといえる．

当研究グループでは，分裂軸の回転に伴い lateral 面から apical 面に露出する TJ 構成蛋白質 claudin を利用した新規がん治療法の開発を目指し，claudin-4 binder を利用したがん治療戦略の構築を試みている[27,28]．

まず，claudin 指向性抗がん剤のモデルとして，claudin-4 結合分子 C-CPE と緑膿菌由来の蛋白質合成阻害因子(PSIF)との融合蛋白質を作製した(図 **3a**)．C-CPE-PSIF は claudin-1, -2, -5 発現 L 細胞では 10 ng/mL でもまったく細胞障害性を示さないのに対して，claudin-4 発現 L 細胞では 1 ng/mL 処理でも顕著な細胞毒性を発揮していた(図 **3b**)．さらに，Caco-2 細胞の単層膜培養系に apical 側，もしくは basal 側から C-CPE-PSIF を添加したとこ

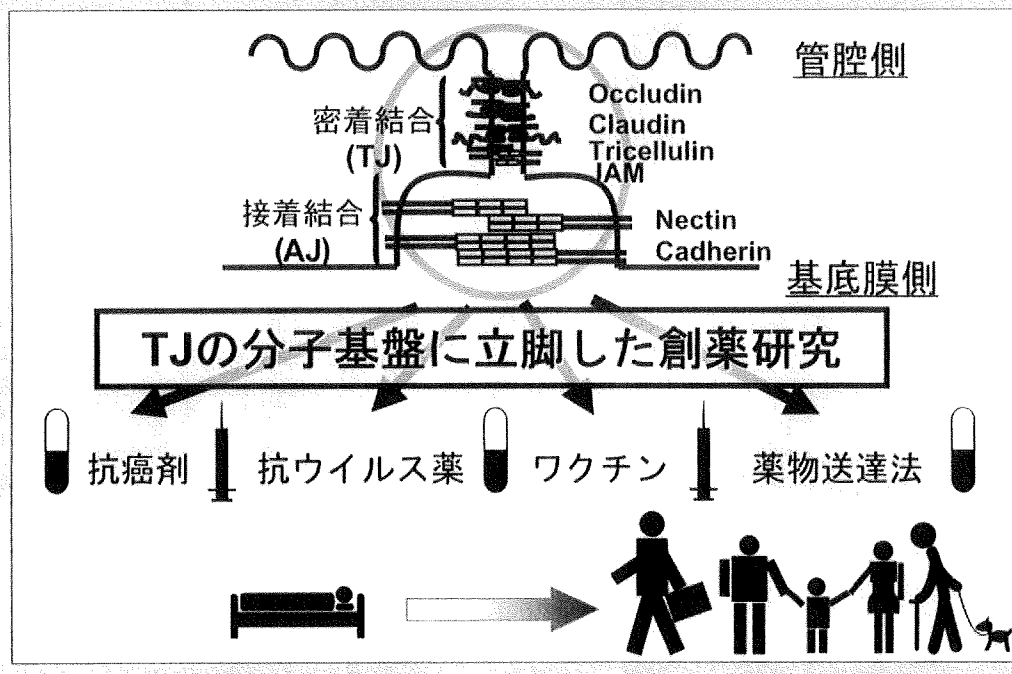

図4　生体バリアを利用した創薬研究

ろ，C-CPE-PSIF を basal 側から加えたときのみ細胞毒性が観察され，C-CPE は claudin の局在性を認識する新しいタイプの抗がん活性分子であると推察された(data not shown)．さらに，マウス乳がん由来細胞株 4T1 細胞を用いて C-CPE-PSIF の抗腫瘍効果を解析したところ，C-CPE-PSIF 処理で *in vitro* および *in vivo* での抗腫瘍活性が認められ体重減少などの副作用は観察されず，claudin-4 結合性を消失させた変異体では *in vitro*，および *in vivo* 抗腫瘍活性がともに消失していた[28]．

これらの結果を踏まえ，現在当研究グループでは，がん化の早期イベント「分裂軸の回転」を標的とした新規がん診断法・治療法の開発を目指し，新規 claudin binder の創出，既存の claudin binder のプローブ化，最適化などを進めている．

まとめ

今回は紙面の都合上割愛させていただいているが，粘膜免疫組織に高発現している claudin を利用したワクチン開発，ウイルスの感染受容体である claudin-1 や occludin を標的とした感染阻害薬の開発などに関しても研究が進展しつつあり，現在までに claudin を利用した非侵襲性投与技術，claudin 指向性抗がん剤，claudin 指向性粘膜ワクチンの創製に成功し，世界に先駆けて生体バリアの分子基盤を利用した創薬戦略の有用性を提唱してきた[19,21,25,28~30]．

周知のように，わが国にはカドヘリンをはじめとした上皮細胞生物学の基礎的知見が集積している．当研究グループでは，本邦固有の上皮細胞生物学の土壌に生まれた創薬研究の萌芽を育み，わが国独自の生体バリアを利用した創薬研究領域を開拓し，本邦発の創薬シーズを一つでも多く“bench side to bed side”へと顕在化できるよう最善を尽くしていきたい(**図4**)．

本稿で紹介した研究成果は，昭和薬科大学渡邊善照教授，藤井まき子准教授，アスビオファーマ株式会社内田博司博士・花田雄志博士をはじめとした多くの先生方のご指導およびご協力，八木ラボおよび渡辺ラボメンバーの知的好奇心および激烈な努力の所産であり，相互作用をいただいたすべての方々に衷心より御礼申し上げます．

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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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Clostridium perfringens enterotoxin

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.

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 receptors 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-disulfophenyl)-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′-GCGGTACCATGGGCGCTGAT GATGTTGTTG-3′, *Kpn*I site is underlined) and a reverse primer (5′-CCTTAATTAATCGCCGTACGCGATTTCCTG-3′, *Pac*I site is underlined). The resulting PCR fragments were subcloned into *Kpn*I/*Pac*I-digested pETH$_{10}$PER (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′-TGGAGGAGGAGGATCTGGAGGAGGAGGA TCTGGAGGATACCCATACGACGTCCCAGACTACGCTAT-3′, *Pac*I site is underlined) and a reverse oligonucleotide (5′-AGCGTAGTCTGGGA CGTCGTATGGGTATCCTCCAGATCCTCCTCCTCCAGATCCTCCTCCTCCA AT-3′, *Pac*I site is underlined). The resulting oligonucleotides were subcloned into *Pac*I-digested pET–DTA–C-CPE, and the sequence was confirmed (pET–DTA-linker-C-CPE).

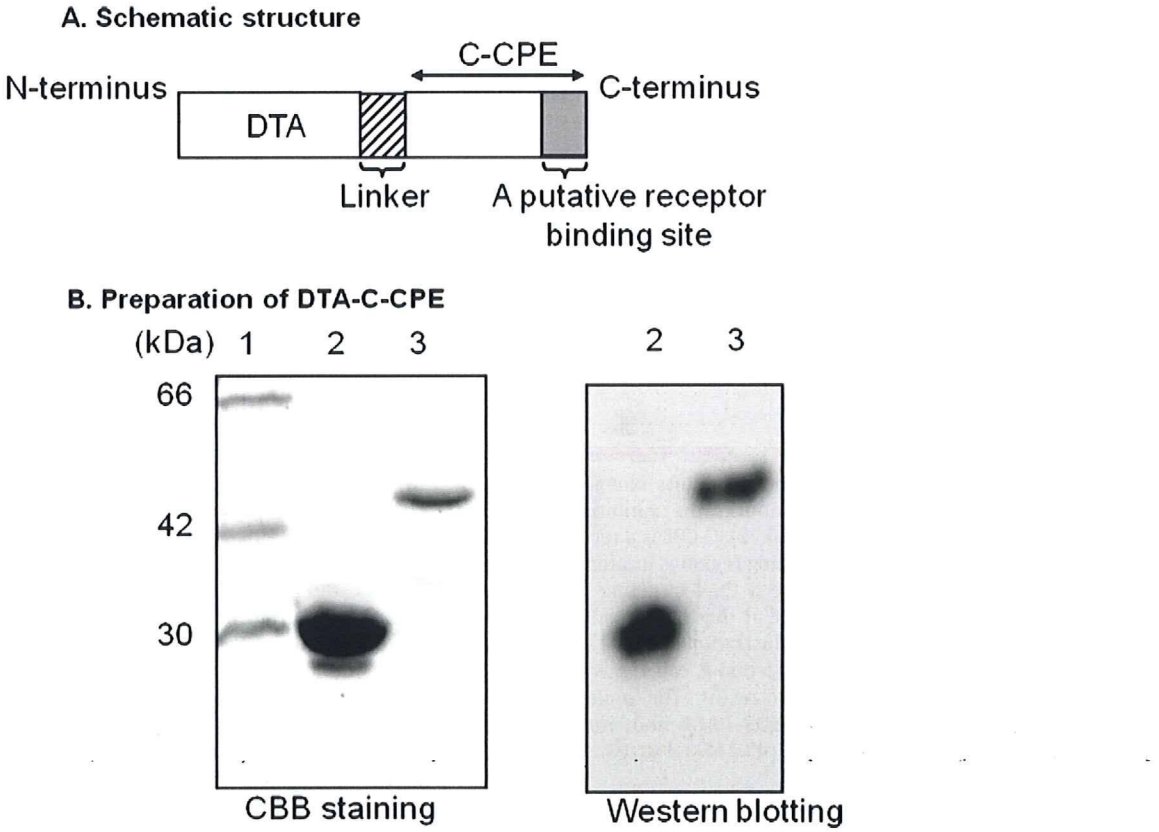

Fig. 1. Preparation of DTA–C-CPE. (A) Schematic structure of DTA–C-CPE. DTA–C-CPE is a fusion protein of DTA and C-CPE with a linker indicated by a slashed column. A dark column indicates a putative receptor-binding region of C-CPE. (B) Preparation of DTA–C-CPE. DTA or DTA–C-CPE was produced by a conventional 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-D-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 $MgCl_2$, 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, 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 CL-4-binding region is located on the C-terminal of C-CPE [23]. To prepare a CL-4-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 CL-4-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 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%.

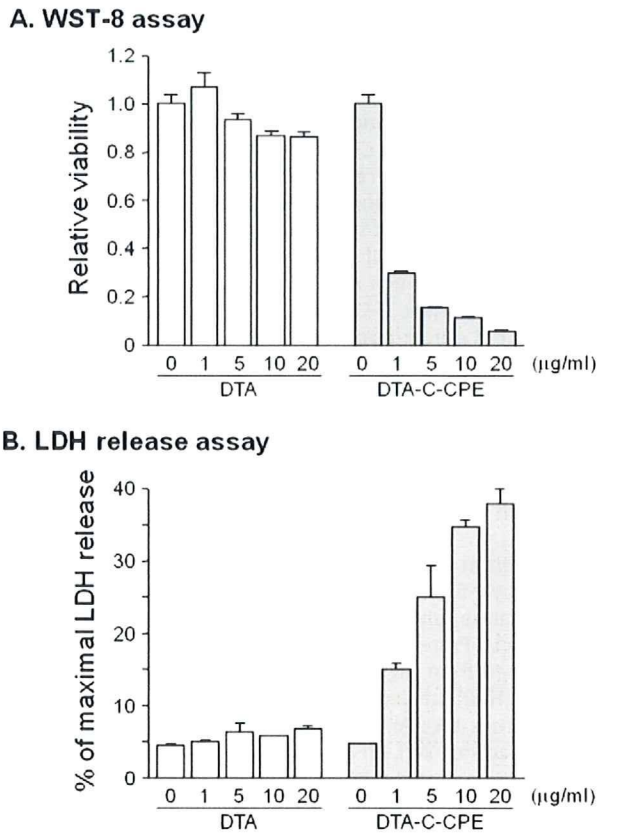

Fig. 2. Cytotoxicity of DTA–C-CPE. CL4/L cells were treated with DTA or DTA–C-CPE at the indicated concentration for 48 h. The cellular viability was measured by WST-8 assay (A) or LDH-release assay (B). Data are the mean ± SD ($n = 3$). The data are representative of three independent experiments.

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 bond 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 CL-4-targeting property to DTA, producing a CL-4-specific cytotoxic agent.

4. Discussion

CL-4 is often overexpressed in some malignant tumors, such as breast, prostate, ovarian, pancreatic and gastric cancers [12,14,17]. CL-4 targeting is a promising method for tumor-targeting therapy. In the present study, we prepared a fusion protein of DTA, a protein

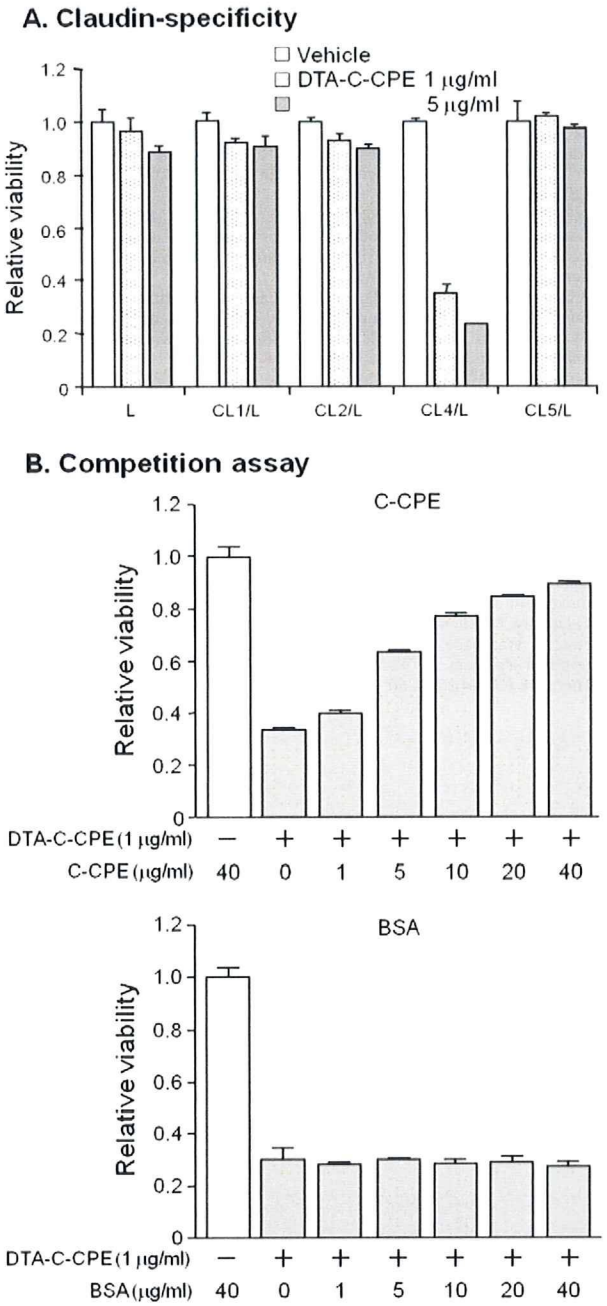

Fig. 3. Cytotoxic properties of DTA–C-CPE. (A) Claudin-specificity. L, CL1/L, CL2/L, CL4/L or CL5/L cells were treated with DTA–C-CPE at the indicated concentration for 48 h. After incubation, the cellular viability was measured by WST-8 assay. Data are the mean ± SD ($n = 3$). The data are representative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

synthesis inhibitory factor, and C-CPE, which binds to CL-4, and we found that the fused protein (DTA–C-CPE) 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 388GMCSF 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-CPE is the receptor-binding domain of CPE, and the CL-4-binding region is located on the C-terminal of C-CPE [15,23,28]. CL-4 has a sorting signal to clathrin-coated vehicles, and CL-4 is expected to be taken up by clathrin-mediated endocytosis [29–31]. CL-4 bound to DTA–C-CPE 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-CPE-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-CPE is a CL ligand. We prepared C-CPE-PSIF, a lead compound for tumor therapy, by using the CL-4-targeting ligand C-CPE [32]. We already determined the functional domains of C-CPE as a CL-4-targeting molecule, and we are using C-CPE 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.

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Mucosal vaccination using claudin-4-targeting

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ABSTRACT

Mucosa-associated lymphoid tissue (MALT) plays pivotal roles in mucosal immune responses. Efficient delivery of antigens to MALT is a critical issue for the development of mucosal vaccines. Although claudin-4 is preferentially expressed in MALT in the gut, a claudin-4-targeting approach for mucosal vaccination has never been developed. In the present study, we found that claudin-4 is expressed in nasal MALT, and we prepared a fusion protein of ovalbumin (OVA) as a model antigen with a claudin-4-binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) (OVA-C-CPE). Nasal immunization with OVA-C-CPE, but not a mixture of OVA and C-CPE, induced the production of OVA-specific serum IgG and nasal, vaginal and fecal IgA. Deletion of the claudin-4-binding region in OVA-C-CPE attenuated the induction of the immune responses. OVA-C-CPE immunization activated both Th1 and Th2 responses, and nasal immunization with OVA-C-CPE showed anti-tumor activity in mice inoculated with OVA-expressing thymoma cells. These results indicate that the claudin-4-targeting may be a potent strategy for nasal vaccination.

1. Introduction

Each year, 17 million people die from infectious diseases worldwide, and 7 million people die from cancers worldwide (http://www.globalhealth.org/infectious_diseases/; http://www.reuters.com/article/healthNews/idUSN16330649200712 17). Thus, the development of methods to prevent and treat infectious diseases and cancers is an important issue for healthcare worldwide. Vaccination against these diseases is a promising approach because of its low frequency of side effects and its great preventative and therapeutic effects. Vaccination strategies are classified as parenteral or mucosal. Parenteral vaccination is effective for the elimination of infectious cells and cancer cells by the induction of systemic immune responses. Parenteral vaccines are administered by injections, which are invasive, painful, and have low levels of patient compliance; moreover, mucosal immunological defense is not induced. In contrast, mucosal vaccine elicits both mucosal and systemic immune responses, resulting in the prevention of infection on the mucosal surfaces and the elimination of pathological cells [1–3]. Mucosal administration is needle-free, less painful, and has improved patience compliance. Thus, mucosal vaccination appears to be an ideal vaccination strategy, although mucosally administered protein antigens are poorly immunogenic. Various approaches for the mucosal delivery of antigens have been investigated [4–6]. Mucosa-associated lymphoid tissues (MALTs) play pivotal roles in mucosal immunological responses [7,8]. MALTs comprise gut-associated lymphoid tissues (GALT), nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT). MALT contains lymphocytes, M cells, T cells, B cells and antigen-presenting cells (APCs), and the efficient delivery of antigens into MALT is essential for mucosal vaccinations [9]. Indeed, there have been several attempts to deliver antigens to MALT using microparticles, liposomes, saponins or chitosans [4–6].

Immunization at one mucosal surface can generate secretory IgA responses at other mucosal sites. Ideally, vaccination at a single site would provide both humoral and cell-mediated protection, not only

Abbreviations: MALT, mucosa-associated lymphoid tissue; OVA, ovalbumin; C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin; OVA-C-CPE, fusion proteins of OVA and C-CPE; GALT, gut-associated lymphoid tissue; NALT, nasopharynx-associated lymphoid tissue; BALT, bronchus-associated lymphoid tissue; APC, antigen-presenting cell; FAE, follicle-associated epithelium; TJ, tight junction; CPE, *Clostridium perfringens* enterotoxin; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; BV, budded baculovirus; FBS, fetal bovine serum; TBS, tris-buffered saline; IFN, interferon; IL, interleukin.

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at the relevant mucosal surface, but also throughout the body [4]. In this regard, nasal vaccination has shown particular potential. Nasally administered vaccines induced mucosal IgA antibody responses in the salivary glands, respiratory tracts, genital tracts, and intestines [10–12]. The nasal route can also induce cytotoxic T lymphocytes in distant mucosal tissues including the female genital tract [13]. Additionally, nasal immunization produced greater systemic antibody responses than other mucosal immunization routes [12,14]. However, despite these encouraging characteristics, free antigens are usually unable to stimulate immune responses following intranasal administration due to their ineffective delivery to immune response-inducing sites [15]. Thus, the effective delivery of antigens to NALT is needed for the development of a potent nasal vaccine.

A single layer of epithelial cell sheet follicle-associated epithelium (FAE) covers NALT. FAE contains M cells, which are key antigen-sampling cells for the delivery of mucosally encountered antigens to the underlying APCs, and FAE plays a pivotal role in the mucosal immunological response [16–18]. Antigen delivery using a ligand for the FAE that covers NALT would be a potent strategy for the development of a mucosal vaccine. Epithelium has well-developed tight junctions (TJs) that seal the intercellular space on the epithelial cell sheets [19,20]. Occludin, claudin and junctional adhesion molecule are components of TJs [21]. Among these components, claudin-4 was preferentially expressed on the dome region of FAE in GALT [22]. We found that claudin-4 was also expressed in NALT (Fig. 1). These findings strongly indicate that claudin-4-targeting may be useful for mucosal vaccines; however, a mucosal vaccine that uses a claudin-4-binder has never been developed.

Clostridium perfringens enterotoxin (CPE) causes food poisoning in humans [23]. A receptor for CPE is claudin-4, and the C-terminal fragment of CPE (C-CPE) is a claudin-4-binder [24–26]. We previously prepared a claudin-4-targeting cytotoxic molecule by genetically fusing a cytotoxin with C-CPE [27,28]. In the present study, we investigated whether claudin-4-targeting is a potent strategy for mucosal vaccine using C-CPE-fused antigen protein.

2. Materials and methods

2.1. Animals

Female BALB/c mice and C57BL/6 mice (6–8 weeks old) were purchased from SLC, Inc. (Shizuoka, Japan). The mice were housed at 23 ± 1.5 °C with a 12-h light/dark cycle and were allowed free access to standard rodent chow and water. After their arrival, the mice were allowed to adapt to their environment for at least 1 week before the experiments. The animal experiments were performed according to the guidelines of Osaka University.

2.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total mRNA was extracted from NALT using Isogen (Nippongene, Toyama, Japan), and the mRNA was reverse-transcribed using an RNA PCR kit (AMV, Ver.3.0) according to the manufacturer's instructions (Takara, Kyoto, Japan). The polymerase chain reaction (PCR) amplification from the resultant cDNA was performed using primer pairs for claudin-4 (forward, 5′-tggatgaactgcgtggtg-3′; reverse, 5′-ggttgtagaagtcgcggatg-3′) for 35 reaction cycles (94 °C, 45 s; 52 °C, 60 s; 72 °C, 30 s) or β-actin (forward, 5′-tagatgggcacagtgtggg-3′; reverse, 5′-ggcgtgatggtgggcatgg-3′) for 30 reaction cycles (94 °C, 30 s; 58 °C, 60 s; 72 °C, 30 s). The amplified products were separated by electrophoresis on a 2% agarose gel and visualized with ethidium bromide.

2.3. Immunoblotting for claudin-4

NALT was lysed in a lysis buffer (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1% Triton X-100, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride). The lysates (10 μg of protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting with anti-claudin-4 (Zymed Laboratory, South San Francisco, CA) or anti-β-actin antibodies (Sigma–Aldrich, St. Louis, MO). The immunoreactive bands were detected with a peroxidase-labeled secondary antibody followed by visualization with a chemiluminescence reagent (Amersham Bioscience, Piscataway, NJ).

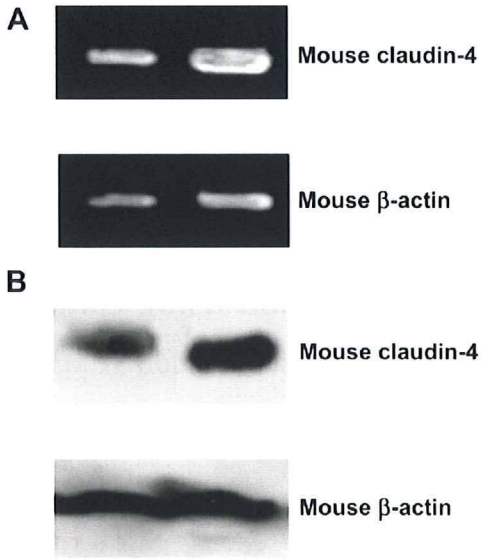

Fig. 1. Expression of claudin-4 in NALT. A) RT-PCR analysis. mRNA was isolated from NALT of mice, and expression of claudin-4 was assayed by RT-PCR. B) Immunoblot analysis. The lysate of NALT was subjected to SDS-PAGE, followed by western blotting with anti-claudin-4 Ab. β-actin was used as an internal control.

2.4. Preparation of OVA-C-CPE fusion proteins

We prepared expression plasmids encoding fusion proteins of OVA with C-CPE or C-CPE303, in which the claudin-4-binding C-terminal 16 amino acids of C-CPE were deleted [29]. Oligonucleotides containing a G4S linker and multiple cloning sites, including KpnI, SpeI, SmaI and PacI sites, were subcloned into NdeI-digested pET16b (Novagen, Darmstadt, Germany), pET-C-CPE and pET-C-CPE303 [30], resulting in pET-MCS and pET-MCS-C-CPEs. OVA cDNA was PCR amplified using pCMV Script/OVA (Kindly provided from Dr. S. Nakagawa, Osaka University, Japan) as a template, a forward primer (5′-gcggtaccatgggctccatcggcgcagc-3′, KpnI site is underlined), and a reverse primer (5′-ccttaattaaaggggaaacacatctgccaa-3′, PacI site is underlined). The resulting OVA fragment was inserted into pET-MCS and pET-MCS-C-CPEs at the KpnI/PacI site, resulting in pET-OVA, pET-OVA-C-CPE and pET-OVA-C-CPE303. The OVA-fusion protein plasmids were transduced into *Escherichia coli* strain BL21 (DE3), and the production of OVA and OVA-C-CPEs was induced by the addition of isopropyl-D-thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris–HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM PMSF, 1 mM 2-mercaptoethanol, and 10% glycerol) supplemented with 8 M urea when necessary. The lysates were applied to HiTrap™ HP (GE Healthcare, Buckinghamshire, UK), and the fusion proteins were eluted with buffer A containing 100–500 mM imidazole. The solvent was exchanged with phosphate-buffered saline (PBS) using a PD-10 column (GE Healthcare), and the purified protein was stored at −80 °C until use. Purification of the fusion proteins was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-his-tag antibody. Protein assays were performed using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with bovine serum albumin as a standard.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Budded baculovirus (BV) displaying mouse claudin-1 or -4 was prepared as described previously [28]. Briefly, the DNA fragments of claudin-1 or -4 were subcloned into the baculoviral transfer vector pFastBac1 (Invitrogen, Gaithersburg, MD). Recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen). Sf9 cells maintained in Grace's Insect medium containing 10% fetal bovine serum (FBS) at 27 °C were infected with the recombinant baculoviruses. After 70 h, the conditioned medium was recovered and centrifuged. The resultant pellets of the BV fraction were suspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail and then stored at 4 °C until use.

The BV displaying claudins was diluted with TBS and adsorbed to the wells of 96-well ELISA plates (Greiner Bio-One, Tokyo, Japan) overnight at 4 °C. The wells were blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharmaceutical, Osaka, Japan) for 2 h at room temperature and the C-CPE, OVA-C-CPE or OVA-C-CPE303 was added. After 2-h incubation, the wells were washed and incubated with anti-his-tag antibody followed by a horseradish peroxidase-conjugated secondary antibody. The immunoreactive proteins were detected using TMB peroxidase substrate at an absorbance of 450 nm.

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2.6. Nasal immunization

Mice were nasally immunized with 10-μl aliquots of OVA, a mixture of OVA and C-CPE, OVA-C-CPE or OVA-C-CPE303 at the indicated schedules. The doses of the proteins were equal to 5 μg of OVA and 1.89 μg of C-CPE.

2.7. OVA-specific antibody production

Seven days after the last immunization, serum and mucosal secretions (nasal washes, vaginal washes, and fecal extracts) were collected. Fecal pellets (100 mg) were suspended in 1 ml of PBS and extracted by vortexing for 10 min. The samples were centrifuged at 3000 × *g* for 10 min, and the resultant supernatants were used as fecal extracts. Vaginal and nasal mucosa were washed with 100 or 200 μl of PBS, respectively.

The titers of OVA-specific antibody in serum, extracts and mucosal washes were determined by ELISA. Briefly, an immunoplate was coated with OVA (100 μg/well in a 96-well plate). Ten-fold serial dilutions of these samples were added to the immunoplate followed by the addition of horseradish peroxidase-conjugated anti-mouse IgG, IgG1, IgG2a or IgA. The OVA-specific antibodies were detected using TMB peroxide substrate. End-point titers were expressed as the dilution ratio, which gave 0.1 above control values obtained for serum of naïve mice at an absorbance of 450 nm.

2.8. Cytokine ELISA

Serum interferon factor-γ (IFN-γ) and Interleukin-13 (IL-13) were measured with an ELISA kit according to the manufacturer's protocol (R&D Systems, Inc., MN).

2.9. Cell cultures

A murine thymoma cell line EL4 (H-2^b) was cultured in RPMI 1640 supplemented with 10% FBS. EG7-OVA cells (OVA-transfected EL4 cells) were maintained in RPMI 1640 containing 10% FBS in the presence of 400 μg/ml of G418.

2.10. Anti-tumor activity

In an anti-tumor assay, female C57BL/6 mice (6–8 weeks) were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA-C-CPE or OVA-C-CPE303 once a week for 3 weeks. All non-vehicle immunizations contained equivalent amounts of OVA (5 μg). Seven days after the last immunization, the mice were subcutaneously inoculated with 1×10^6 EG7-OVA cells. Tumor growth was monitored by measuring two diameters, and the tumor volume was calculated as $a \times b \times b/2$, where a is the maximum diameter of the tumor and b is the minimum diameter of the tumor.

2.11. Statistical analysis

Results were analyzed by an analysis of variance (ANOVA) followed by the Dunnett multiple comparison test, and statistical significance was assigned at $p < 0.05$.

3. Results

3.1. Expression of claudin-4 in NALT

Nasal vaccine is a potent therapy for infectious diseases and cancers since nasal vaccination potentiates humoral and cellular immune responses throughout the body. NALT is the nasal lymphoid tissue, and effective delivery of antigens to NALT is critical for the development of mucosal vaccinations. A previous report showed that claudin-4 is expressed in GALT [22], whereas it is unclear whether claudin-4 is expressed in NALT. To investigate the expression of claudin-4 in NALT, NALT was isolated from mice, and the NALT lysate was subjected to RT-PCR and immunoblotting analyses. As shown in Fig. 1A and B, claudin-4 mRNA and protein were detected in NALT. These data indicate that claudin-4-binder may be a targeting molecule for NALT.

3.2. Preparation of claudin-4-targeting OVA

Claudin has low antigenicity, and there has been little success in the preparation of antibodies against the extracellular region of claudin. C-CPE corresponding to aa 184–319 at the C-terminal of CPE is a claudin-4-binder [24,25]. We previously prepared a claudin-4-targeting cytotoxic molecule genetically fused with C-CPE [27]. To evaluate whether a claudin-4-targeting strategy is an effective method for mucosal vaccination, we genetically fused C-CPE with OVA, a popular model antigen for vaccination, to yield OVA-C-CPE (Fig. 2A). OVA-C-CPE was produced by *E. coli* and purified by affinity chromatography. Purification of the protein was confirmed by SDS-PAGE and immunoblotting (Fig. 2B). The molecular size was identical to the predicted size of 62 kDa for OVA-C-CPE. To evaluate the binding of OVA-C-CPE to claudin-4, we performed ELISA with a claudin-displaying BV-coated immunoplate. OVA-C-CPE or C-CPE was added to wells coated with wild-type BV, claudin-1-BV or claudin-4-BV. The bound proteins were detected using anti-his-tag antibody. Like C-CPE, OVA-C-CPE bound to claudin-4-BV but not wild-type BV or claudin-1-BV (Fig. 2C).

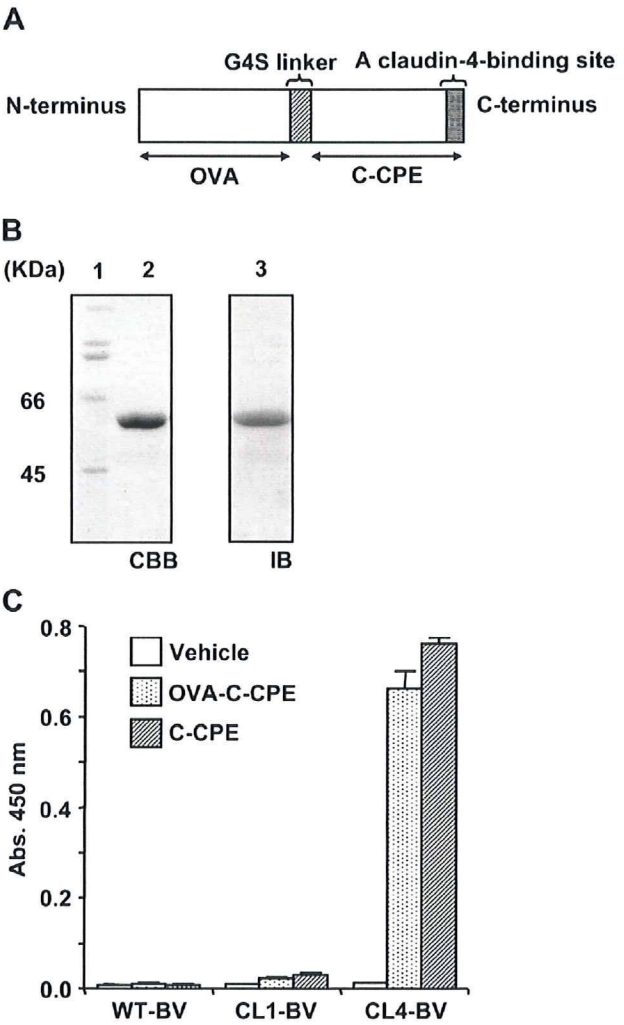

Fig. 2. Preparation of OVA-C-CPE. A) Schematic illustration of OVA-C-CPE. The claudin-4-binding site of C-CPE is located in the C-terminal 16 amino acids [29]. OVA was fused with C-CPE at the N-terminal of C-CPE, resulting in OVA-C-CPE. B) Purification of OVA-C-CPE. OVA-C-CPE was expressed in *E. Coli* as a his-tagged protein and isolated by Ni-affinity chromatography. The purification of OVA-C-CPE was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB, left panel) and by immunoblotting with an anti-his-tag antibody (IB, right panel). Lane 1: molecular weight marker; lane 2, 3: OVA-C-CPE. The putative molecular mass of OVA-C-CPE is 62 kDa. C) Binding of OVA-C-CPE to claudin-4. Wild-type BV (WT-BV), BV displaying claudin-1 (CL1-BV) or -4 (CL4-BV) was absorbed onto a 96-well immunoplate, and then vehicle, OVA-C-CPE or C-CPE was added to the well. OVA-C-CPE or C-CPE bound to BV was detected by an anti-his-tag Ab followed by horseradish peroxidase-labeled secondary Ab. C-CPE was used as a positive control for a claudin-4-binding. Data are means ± SD ($n = 4$).

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3.3. Induction of OVA-specific humoral responses

To clarify whether claudin-4-targeting activates an immune response, we investigated antigen-specific humoral responses at both systemic and mucosal sites in mice that received nasally administered OVA-C-CPE. Mice received an intranasal administration of OVA, a mixture of OVA and C-CPE, or OVA-C-CPE fusion protein once a week for 3 weeks. Seven days after the last administration, we measured the OVA-specific serum IgG, nasal IgA, vaginal IgA and fecal IgA levels. As shown in Fig. 3A, the OVA-specific serum IgG responses were increased in mice immunized with OVA-C-CPE as compared to the mice immunized with OVA or a mixture of OVA and C-CPE. The OVA-specific IgA responses in nasal washes were greater from mice immunized with OVA-C-CPE than from mice immunized with OVA or a mixture of OVA and C-CPE (Fig. 3B). It is a superior character of mucosal vaccination that antigen-specific IgA responses were induced not only at the immunized site but also at remote mucosal surfaces [4]. As shown in Fig. 3C and D, nasal immunization with OVA-C-CPE activated vaginal and fecal OVA-specific IgA responses. The OVA-specific IgA responses did not occur in mice immunized with a mixture of OVA and C-CPE. These data suggest that fusion of OVA with C-CPE is critical for successful nasal vaccination.

We previously found that the C-terminal 16 amino acids of C-CPE are essential for claudin-4-binding [29]. To investigate the involvement of claudin-4 in OVA-specific humoral responses in mice nasally immunized with OVA-C-CPE, we prepared OVA-C-CPE303, in which the claudin-4-binding region was deleted (Fig. 4A). Deletion of the 16 amino acid region attenuated the claudin-4-binding of OVA-C-CPE (Fig. 4B). OVA-specific serum IgG and nasal, vaginal and fecal mucosal IgA responses were also attenuated in mice immunized with OVA-C-CPE303 (Fig. 4C and D, 4E and 4F, respectively). No histological mucosal injury was found after nasal immunization with OVA-C-CPE (data not shown). These findings indicate that claudin-4-targeting may be involved in nasal vaccination by OVA-C-CPE.

3.4. Induction of Th1 and Th2 responses by OVA-C-CPE

Nasal immunization of antigen induced antigen-specific immune responses including Th1- and Th2-type responses [31,32]. We next investigated whether nasal immunization with OVA-C-CPE evoked Th1- or Th2-type responses. The OVA-specific IgG1 (a Th2 response) and IgG2a (a Th1 response) responses in the serum of mice nasally immunized with OVA-C-CPE were significantly enhanced compared to those of mice immunized with OVA alone or a mixture of OVA and C-CPE (Fig. 5A). Measurement of Th1 (IFN-γ) and Th2 (IL-13)-specific cytokines in splenocytes isolated from mice nasally immunized with OVA, a mixture of OVA and C-CPE, or OVA-C-CPE showed that nasal immunization with OVA-C-CPE increased both Th1 and Th2 cytokine production (Fig. 5B). Th1 and

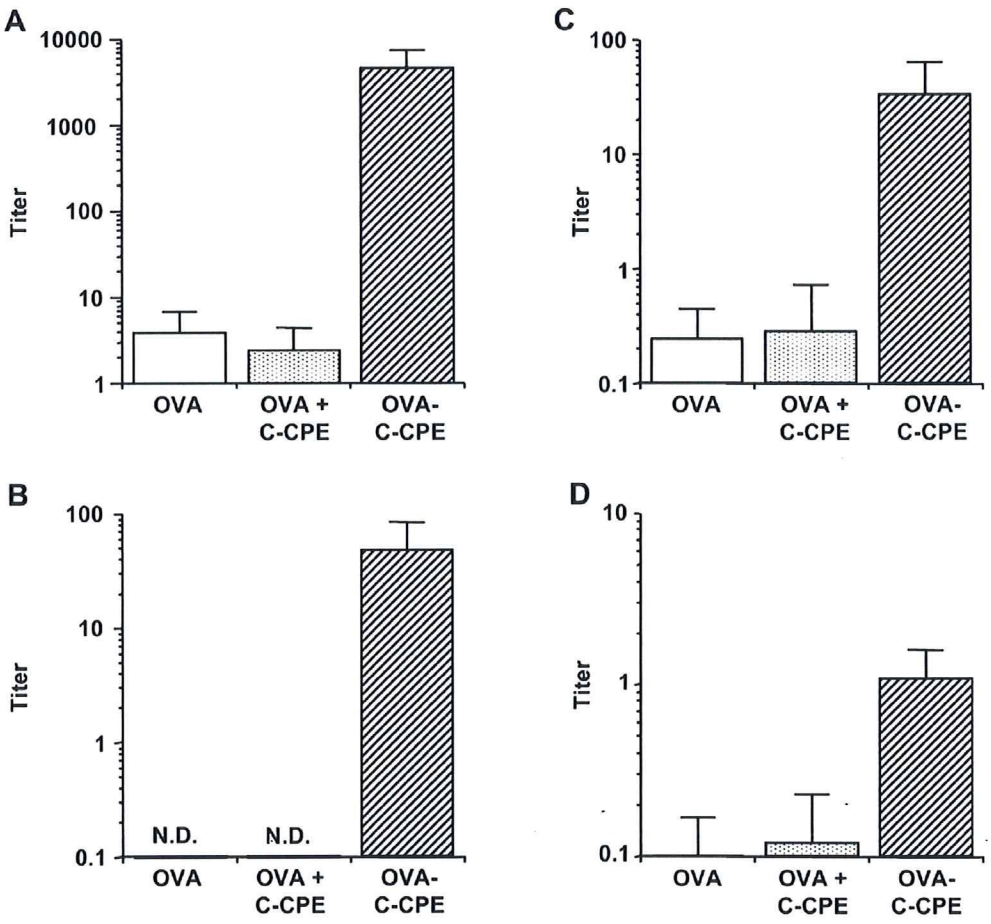

Fig. 3. Production of OVA-specific IgG and IgA by OVA-C-CPE. Mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, or OVA-C-CPE (5 μg OVA) once a week for 3 weeks. Seven days after the last immunization, the levels of serum IgG (A), nasal IgA (B), vaginal IgA (C) and fecal IgA (D) were determined by ELISA. Data are means ± SD ($n = 4$). The results are representative of three independent experiments. N.D., not detected.

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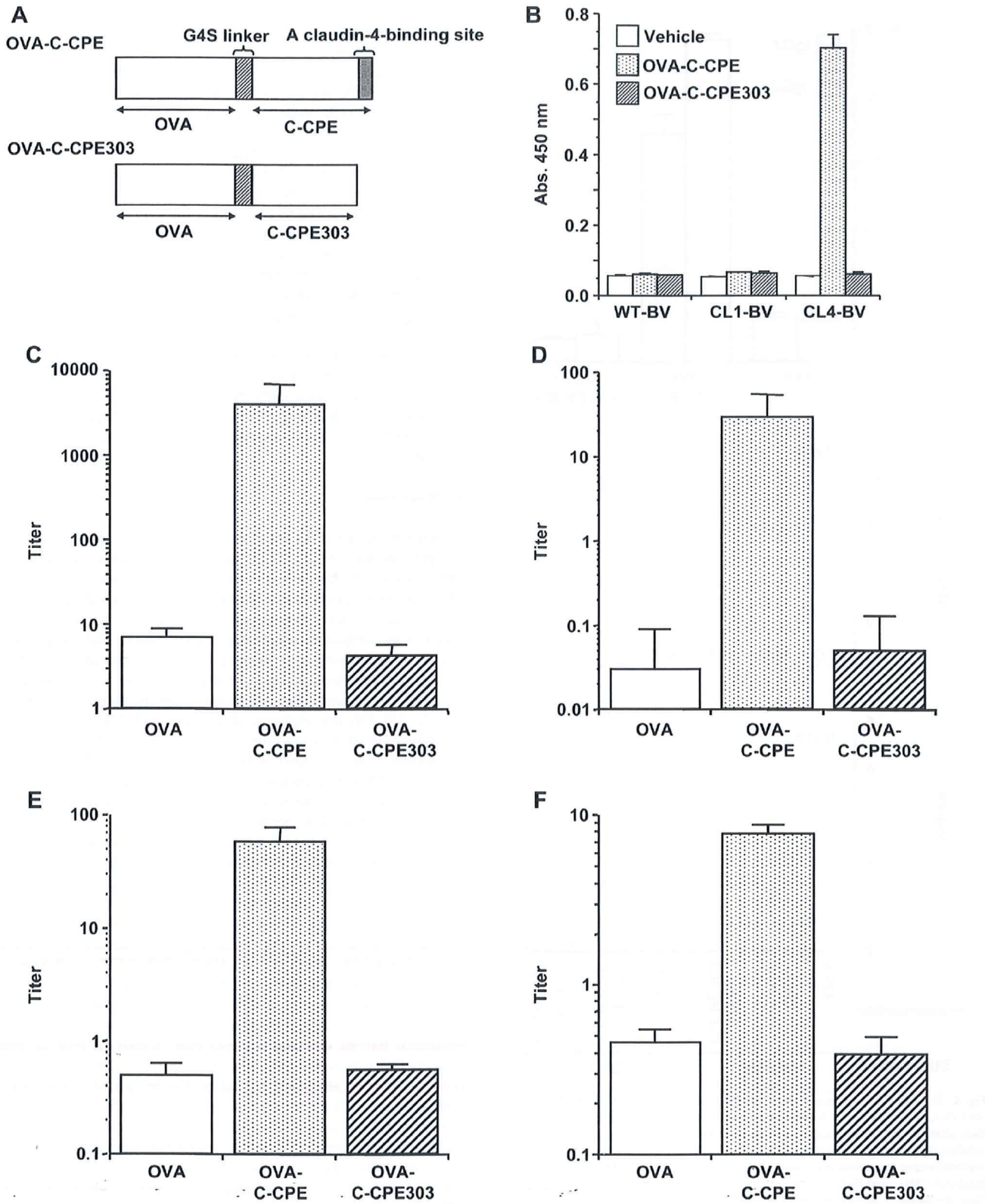

Fig. 4. Involvement of claudin-4 in the immune responses to OVA-C-CPE. A) Schematic illustration of OVA-C-CPE mutant. The C-terminal 16 amino acid-deleted C-CPE mutant (C-CPE303) did not bind to claudin-4 [29]. To clarify the involvement of claudin-4 in the immune response initiated by OVA-C-CPE, OVA was fused with C-CPE303, resulting in OVA-C-CPE303. B) Interaction of OVA-C-CPE303 with claudin-4. Binding of OVA-C-CPE303 to claudin-4 was investigated by ELISA with wild-type BV (WT-BV), claudin-1 or -4-displaying BV (CL1-BV, CL4-BV). C) Immune responses by OVA-C-CPE303. Mice were nasally immunized with OVA, OVA-C-CPE or OVA-C-CPE303 (5 μg OVA) once a week for 3 weeks. Seven days after the last immunization, the levels of serum IgG (C), nasal IgA (D), vaginal IgA (E) and fecal IgA (F) were measured by ELISA. Data are means ± SD ($n = 4$). Data are representative of three independent experiments.

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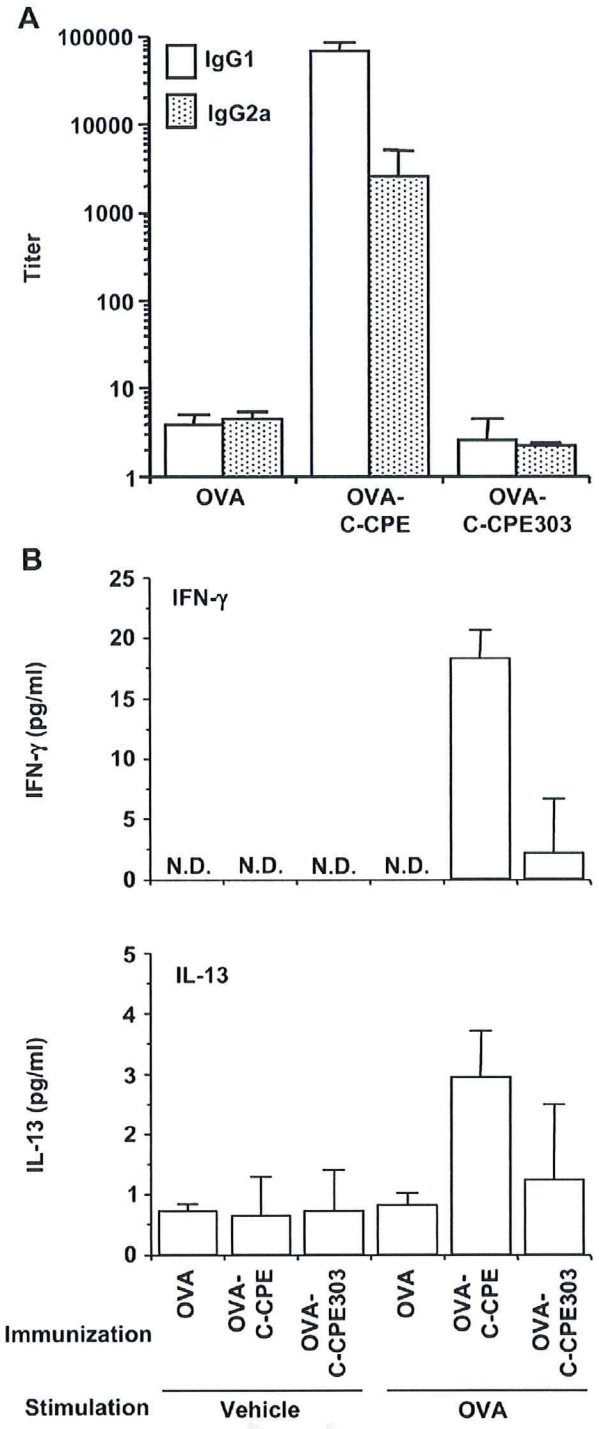

Fig. 5. Th1 and Th2 responses induced by OVA-C-CPE. Mice were nasally immunized with OVA, OVA-C-CPE or OVA-C-CPE303 (5 μg of OVA) once a week for 3 weeks. Seven days after the last immunization, serum and splenocytes were collected. Serum IgG subclass (IgG1 and IgG2a) was determined by ELISA (A). The splenocytes isolated from the immunized mice were stimulated with vehicle or OVA (1 mg/ml) for 24 h, and the cytokines (IFN-γ and IL-13) in the conditioned medium were measured by ELISA (B). Data are means ± SD ($n = 4$). N.D., not detected.

Th2 responses in IgG production and cytokines production were not observed in mice nasally immunized with OVA-C-CPE303 (Fig. 5A and B). These data indicate that claudin-4-targeting may be a potent method for mucosal vaccination.

3.5. Anti-tumor immune response induced by the claudin-4-targeting vaccine

To evaluate the immune responses induced by nasal vaccination with OVA-C-CPE, we performed an in vivo anti-tumor assay with EG7 thymoma cells, which are syngeneic tumor cells derived from OVA cDNA-transfected EL4 thymoma cells [33]. C57BL/6 mice were immunized with vehicle, OVA, a mixture of OVA and C-CPE, or OVA-C-CPE once a week for 3 weeks. Seven days after the last immunization, mice were challenged with EG7 thymoma cells. Tumor growth was monitored by calculating the average tumor volume. As shown in Fig. 6A, tumor growth was significantly suppressed in mice immunized with OVA-C-CPE, whereas the tumor growth was not suppressed in mice immunized with OVA or a mixture of OVA and C-CPE. Immunization with OVA-C-CPE303, in which the claudin-4-binding region was deleted, did not induce a protective immune response against tumor challenge. Immunization with OVA or OVA-C-CPE303 did not stimulate Th1- and Th2-immune responses including IgG1, IgG2a, IFN-γ and IL-13 production; whereas immunization with OVA-C-CPE stimulated these immune responses (Fig. 6B and C). These data indicate that nasal immunization with a claudin-4-targeting vaccine may be useful for cancer therapy.

4. Discussion

Recent progress in vaccine development has provided new insight into vaccine therapies for not only infectious diseases but also cancer, Alzheimer disease and Parkinson disease [3,34]. Mucosal vaccination, such as oral, nasal and pulmonary immunization, has greater therapeutic potential and increased patient comfort as compared to parenteral vaccination. The nasal cavity is the most promising site since it has low enzymatic activity and highly available immunoreactive sites; however, immunoresponses are not stimulated by intranasal administration of antigens [15,35–37]. Efficient delivery of antigens to NALT is critical for the development of nasal vaccines. In the present study, we found that intranasal immunization with antigen fused with a claudin-4-binder, C-CPE, stimulated humoral and mucosal immune responses and that these immune responses did not occur when the claudin-4-binding domain was deleted.

How does OVA-C-CPE activate immune responses? Claudin plays a pivotal role in the TJ-barrier in epithelium [38]. We previously found that C-CPE modulates the claudin-4 barrier and enhances mucosal absorption of dextran [30]. Activation of immune responses by OVA-C-CPE may be caused by modulation of the epithelial barrier in NALT, resulting in the uptake of OVA-C-CPE or its degradable product into NALT. OVA-C-CPE modulated the epithelial barrier in a human intestinal model of Caco-2 monolayer cells (data not shown). C-CPE enhanced jejunal absorption of dextran with a molecular mass of 4–20 kDa, and the integrity of the epithelial barrier in nasal mucosa was similar to that in jejunal mucosa [30,39]. OVA-C-CPE, which has a molecular mass of 62 kDa, may be poorly absorbed by nasal tissue. When OVA-C-CPE is degraded into fragments with a molecular mass of less than 20 kDa, the OVA fragment might be absorbed across nasal epithelium. A mixture of OVA and C-CPE did not induce an immune response, and deletion of the claudin-4-binding region in OVA-C-CPE attenuated the immune responses caused by nasal immunization with OVA-C-CPE. These findings indicate that targeting to claudin-4 rather than modulating the claudin-4 barrier by C-CPE is involved in the immune response to nasal vaccinations of OVA-C-CPE.

What cells are taken up OVA-C-CPE? NALT is covered by a unique epithelial layer known as FAE. Lymphocytes, T cells, B cells and APCs underlie the FAE. Antigen presentation to the

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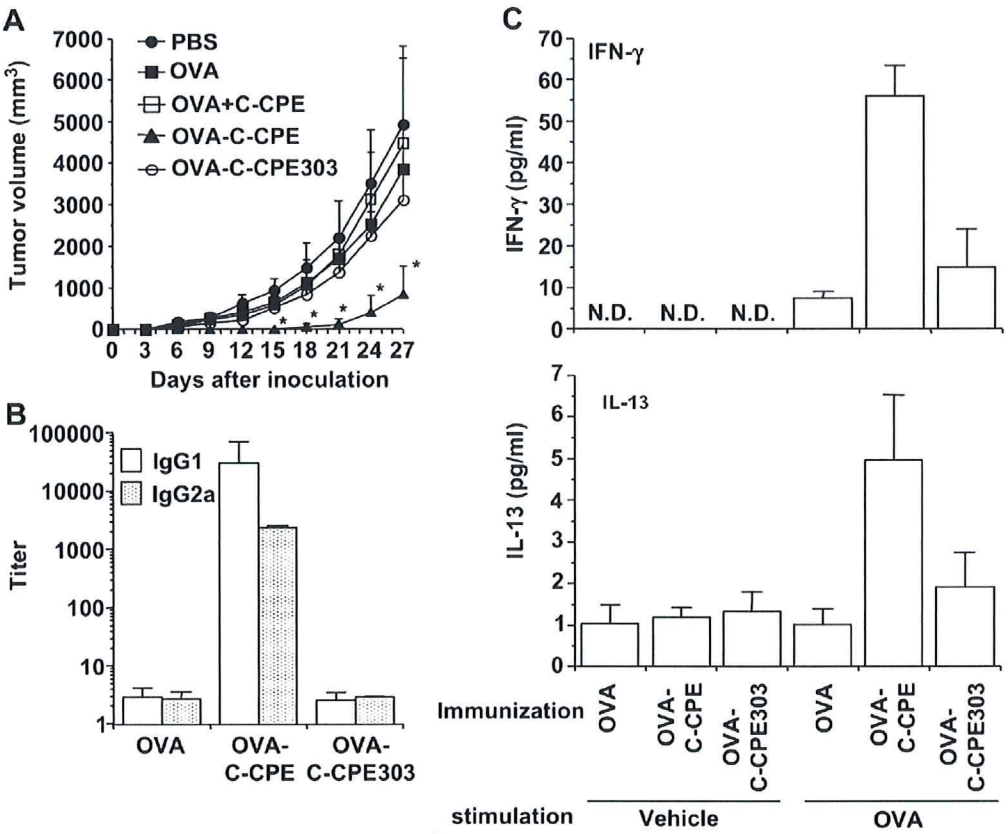

Fig. 6. Anti-tumor activity induced by immunization with OVA-C-CPE in an EG7 cancer model. A) Protective immune response against tumor challenge. C57BL/6 mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA-C-CPE, or OVA-C-CPE303 (5 μg of OVA) once a week for 3 weeks. Seven days after the last immunization, the mice were injected s.c. on the right back with 1×10^6 EG7 cells. The tumor volumes were calculated as described in the Materials and methods. Data are means ± SD ($n = 4$). The results are representative of two independent experiments. *Significantly different from the vehicle-immunized group ($P < 0.05$). B, C) Immune responses in the cancer model. Mice were nasally immunized with vehicle, OVA, OVA-C-CPE, or OVA-C-CPE303 (5 μg of OVA) once a week for 3 weeks. Seven days after the last immunization, the serum and splenocytes were recovered. Serum IgG subclass (IgG1 and IgG2a) was determined by ELISA (B). The splenocytes were stimulated with vehicle or OVA (1 mg/ml) for 24 h, and the cytokines (IFN-γ and IL-13) in the conditioned medium were measured by ELISA (C). Data are means ± SD ($n = 4$). N.D., not detected. The results are representative of two independent experiments.

immunocompetent cells by FAE is a trigger of mucosal immune responses [40,41]. Claudin-4 is expressed in the FAE of MALT [22]. Claudin-4 contains clathrin-sorting signal sequences in its C-terminal intracellular region [42,43]; thus, it may be taken up by clathrin-mediated endocytosis. Indeed, Matsuda et al. (2004) showed the endocytosis of claudins during the remodeling of TJs [44], and a C-CPE-fused molecule was intracellularly taken up [27]. OVA-C-CPE may be taken up into FAE followed by the presentation of antigens to the underlying immunocompetent cells. The FAE is enriched with specialized antigen-sampling epithelial cells known as M cells. M cells form an apparent pocket at the basal membrane site, and this pocket contains T cells, B cells, macrophages and dendritic cells. M cells deliver samples of foreign material by active transepithelial vesicular transport from the lumen directly to intraepithelial lymphoid cells and to subepithelial organized lymphoid tissue [6,16,40]. An antigen delivery system to M cells has been developed, and ligands for M cells, including a lectin, a peptide or a specific antibody, have been used for mucosal vaccination [45–48]. It has not been determined if claudin-4 is expressed in M cells and if OVA-C-CPE is taken up into M cells. Further investigation to clarify the mode of action of the claudin-4-targeting vaccine is needed.

Safety is essential for clinical application of the claudin-4-targeting vaccine. Histological injury was not detected after the administration of OVA-C-CPE. C-CPE is the receptor-binding domain of CPE without the cytotoxic domain [24,49]. Claudin functions as an epithelial barrier between the inside and the outside of the body, and modulation of the claudin-4 barrier by the claudin-4 binder may cause side effects due to the non-specific influx of xenobiotics through the loosened epithelial barrier. The claudin family contains more than 20 members, and the claudin expression and barrier-function differ among tissues [38,50]. Expression profiles of claudin in the mucosal epithelium also differ among the sites of epithelium [51,52]. To reduce the risk of solute influx, further investigation of the difference in claudin expression between MALT and the other sites is important. Preparation of a claudin binder with less modulation of the epithelial barrier is also needed.

In rodents, NALT is found on both sides of the nasopharyngeal duct dorsal to the cartilaginous soft palate. Humans do not have NALT, except at an early age [53]; but, they possess oropharyngeal lymphoid tissues, including unpaired nasopharyngeal tonsils (adenoids) and bilateral tubular palatine, and lingual tonsils (Waldeyer's ring), which seem to correspond functionally to NALT [7,54]. The expression of claudin-4 in the human MALT, such as the tonsils and adenoids, should be investigated for the development of oral mucosal vaccine.

5. Conclusions

In the present study, we prepared C-CPE-fused OVA, and we found that the intranasal administration of the fusion protein increased not

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only nasal IgA levels but also OVA-specific serum IgG, vaginal IgA and fecal IgA levels. Moreover, deletion of the claudin-4-binding region in the fusion protein caused the loss of immunomodulating activities. The claudin-4-targeting antigen immunization activated both Th1 and Th2 responses and showed anti-tumor activity in mice inoculated with OVA-expressing thymoma cells. This is the first report to indicate that claudin-4-targeting may be a promising strategy for the development of mucosal vaccines.

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