and claudin-4) were changed to alanines, resulting in C-CPE$_{Y306A/L315A}$-PSIF (14). C-CPE-PSIF mediated dose-dependent cytotoxicity in 4T1 cells, reaching 63% cell death at 100 ng/ml. In contrast, C-CPE$_{Y306A/L315A}$-PSIF was not cytotoxic even at 500 ng/ml, indicating that the cytotoxicity of C-CPE-PSIF in 4T1 cells may be mediated by its binding to claudin-4. To investigate the in vivo antitumor activity of C-CPE-PSIF, 4T1 cells (2 x 10^6 cells) were inoculated into the right flank of mice on day 0. Vehicle, C-CPE, C-CPE-PSIF or C-CPE$_{Y306A/L315A}$-PSIF at a dose of 5 μg/kg was intratumorally injected on days 2, 4, 7, 9, 11, and 14. C-CPE-PSIF significantly suppressed tumor growth, and the tumor volume in the C-CPE-PSIF-treated group was 36% of that in the vehicle-treated group on day 16. In contrast, C-CPE and C-CPE$_{Y306A/L315A}$-PSIF, which lacked claudin-4-binding activity, had no effect on tumor growth, indicating that the antitumor activity of C-CPE-PSIF may depend on claudin-4 targeting (13).

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

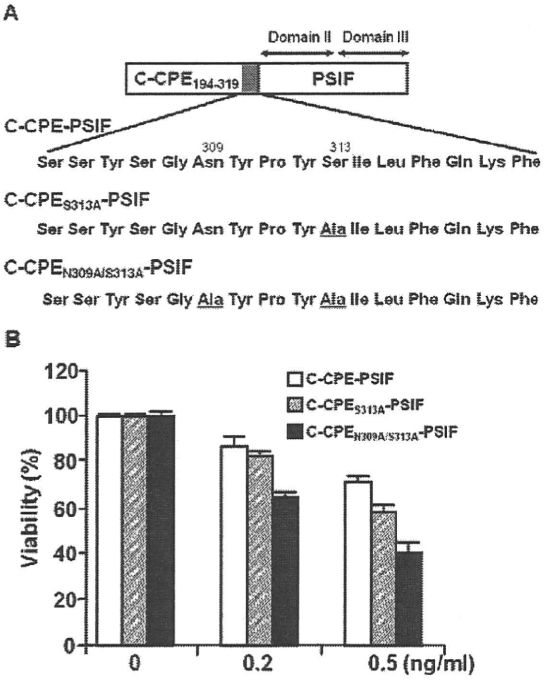

Figure 1. Cytotoxicity of C-CPE-PSIF fusion proteins (A) Schematic illustration of C-CPE-PSIF fusion proteins. C-CPE is the C-terminal fragment of CPE corresponding to amino acids 194-319. The dark area indicates the putative claudin-4-binding region (15). Changing Asn to Ala at position of 309 or Ser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

Perspective of claudin-targeted tumor therapy

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

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

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

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

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

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

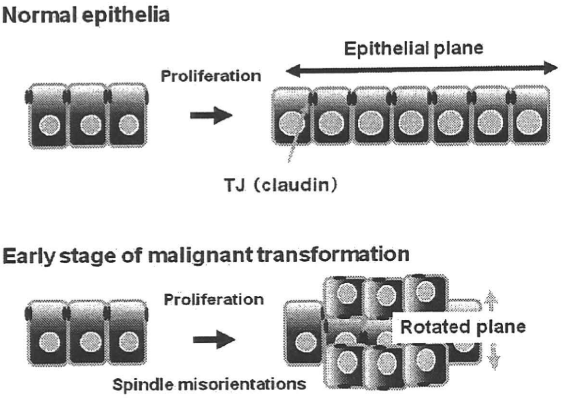

Figure 2. Scheme of malignant transformation in epithelium. In normal epithelial cells, the cells are divided in parallel with the epithelial plane. In the early stage of epithelial tumorigenesis, the orientation of the mitotic spindles is deregulated, and the cells proliferate by out-of-plane division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

Conflicts of Interest

No potential conflicts of interest to disclose.

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≪若手研究者紹介≫

生体バリアの分子基盤を利用した創薬研究

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1. はじめに

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

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

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

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2. これまで

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

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

Claudinは分子量～23 kDaの4回膜貫通蛋白質であり，24種類の分子からなるファミリーを形成している．興味深いことに発現およびバリア機能には組織特異性が認められ，claudin-1は皮膚バリア，claudin-5は血液脳関門バリアを担っている．さらに，ヒトでは12種類の癌において発現異常が認められること，粘膜免疫組織に高発現していること，ウイルスの感染受容体としても機能していることから，claudinを標的とした薬物吸収促進法，癌ターゲティング法，粘膜ワクチン，抗ウイルス薬の開発など，新たな創薬研究領域創成の可能性が強く示唆された．しかしながら，claudinは抗原性が低い上に立体構造が解析されておらず，抗体を含めてclaudin binderの創製は著しく立ち遅れており，claudinを利用した創薬研究は皆無に等しいのが現状であった．

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

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

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

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

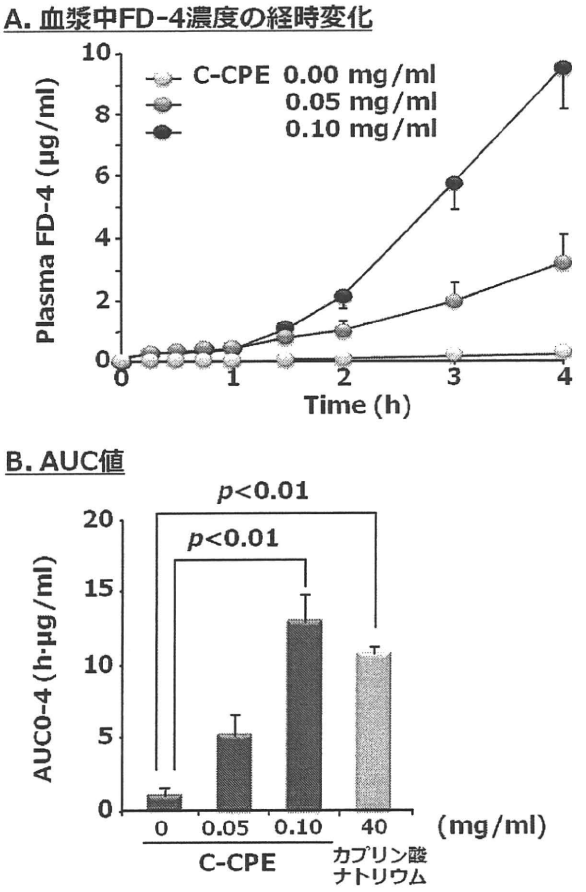

図1　C-CPEの腸管吸収促進活性
FITCラベルした分子量4000のデキストラン（FD-4）をモデル薬物として用いて，ラット腸管ループ法によりC-CPEの吸収促進活性を解析（文献1を一部改変）．

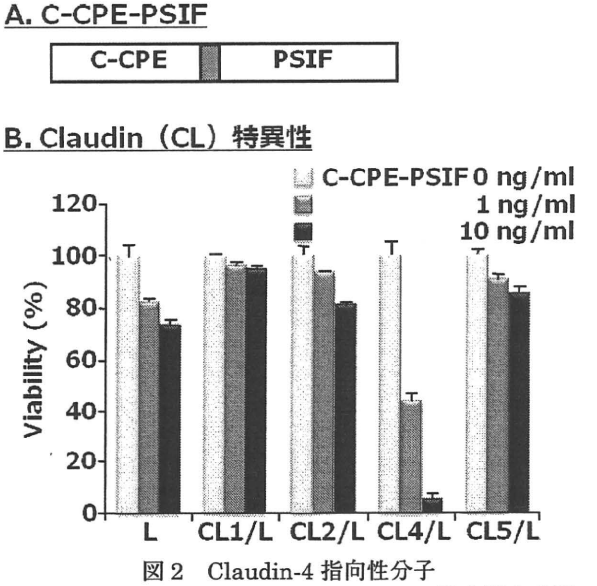

図2　Claudin-4指向性分子
Pseudomonas aeruginosa exotoxinの蛋白質合成阻害ドメイン（PSIF）とC-CPEとの融合体（A）を作製し，各種claudin発現細胞に各濃度24時間処理後にWSTアッセイにより細胞毒性を解析（B）．

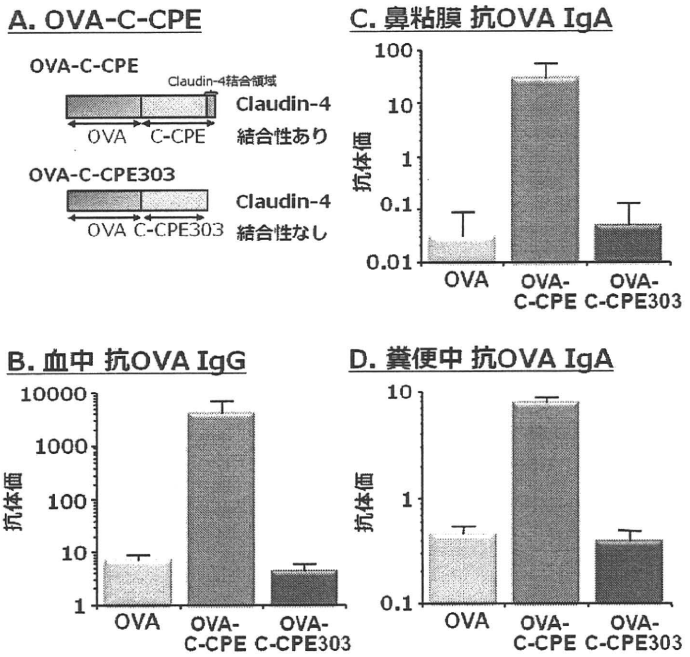

図3　Claudin binderを利用した粘膜ワクチン
モデル抗原（卵白アルブミン：OVA）とC-CPEとの融合体（A）を作製し，マウスに経鼻投与し，血中(B)，鼻粘膜洗浄液(C)，糞便抽出液(D)に含まれるOVA特異的抗体価を解析．

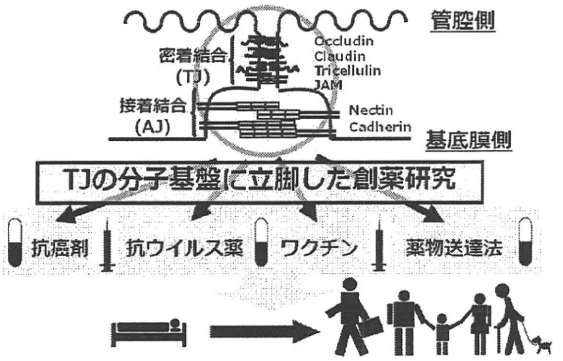

図4　生体バリアを利用した創薬研究

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

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

3. これから

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

三つ子の魂百までというように，「細胞を敬え，細

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

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

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

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

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

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

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ABSTRACT

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

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

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

Freeze-fracture replica microscopy analysis revealed that TJs form a series of continuous strands within plasma membranes (Staehelin, 1973). TJ strands from the membranes of adjacent cells form a paired strand, and the paired strands seal the intercellular space between the adjacent cells. The TJ strands are composed of integral membrane proteins,

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ABBREVIATIONS: CPE, *Clostridium perfringens* enterotoxin; C-CPE, C-terminal fragment of CPE; PSIF, protein synthesis inhibitory factor; C-CPE-PSIF, C-CPE-fused PSIF; CL4-B16, claudin-4-expressing B16; TJ, tight junction; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; ADR, adriamycin.

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

Clostridium perfringens enterotoxin (CPE), a 35-kDa polypeptide, causes food poisoning in humans. CPE binds to its receptor, and then causes changes in the membrane permeability by complex formation on the plasma membrane followed by the induction of oncosis and apoptosis (McClane and Chakrabarti, 2004). The local administration of CPE suppresses solid tumor growth (Michl et al., 2001; Kominsky et al., 2004; Santin et al., 2005); however, whole CPE had never been applied into a ligand for claudin CPE receptors because of its strong cytotoxicity. The receptor-binding region of CPE (C-CPE) can be used for claudin-targeted cancer therapy (Saeki et al., 2009). Immunotoxins, consisting of a protein toxin connected to a binding ligand, such as an antibody or growth factor, have been developed and used for clinical therapy. Protein synthesis inhibitory factor (PSIF) derived from *Pseudomonas* exotoxin is a widely used protein toxin (Kreitman and Pastan, 2006), and intratumoral administration of the claudin-4-targeting PSIF has been shown to attenuate solid tumor growth (Saeki et al., 2009). In the present study, we investigated whether claudin CPE receptors can be a target for tumor metastasis by using the claudin-4-targeting PSIF as a claudin-targeting agent.

Materials and Methods

Cell Culture. Mouse melanoma cell line B16-BL6 and mouse breast cancer cell line 4T1 were cultured in modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and 10 mmol/ml HEPES, respectively. The cells were maintained in a 5% CO_2 atmosphere at 37°C.

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

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

Cell Proliferation Assay. B16 or CL4-B16-expressing cells (CL4-B16 cells) (2×10^4 cells) were seeded into a 24-well plate. At the indicated time points, the cells were stained with trypan blue, and the number of viable cells was counted by using a hemocytometer under a microscope.

In Vitro Metastasis Analysis. A cell culture insert with an 8-μm pore size membrane (BD Biosciences Discovery Labware, Bedford, MA) was used for the invasion assay. The upper surface of the chamber was coated with 50 μl of 0.1 mg/ml Matrigel (BD Biosciences, San Jose, CA), and the lower chamber was filled with DMEM containing 10% FBS. Cells were cultured to approximately 80% confluence and serum-starved overnight (0.5% FBS). Then, cells (1×10^5 cells) suspended in DMEM with 0.5% FBS were placed into the upper chamber and incubated at 37°C for 24 h. After incubation, noninvading cells were removed, and the membrane was stained with Diff-Quick reagent. The stained cells in five randomly selected fields were counted under a microscope.

Preparation of C-CPE-PSIF. The C-terminal fragment of CPE (C-CPE)-fused PSIF (C-CPE-PSIF) was prepared as described previously (Saeki et al., 2009). In brief, pET-C-CPE-PSIF was transduced into *Escherichia coli* BL21 (DE3) (Novagen, Darmstadt, Germany), and the production of C-CPE-PSIF was induced by the addition of 0.25 mM isopropyl-D-thiogalactopyranoside. The cells were harvested and then lysed in buffer A [10 mM Tris-HCl (pH 8.0), 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol]. The lysates were centrifuged, and the resultant supernatant was applied to HiTrap Chelating HP (GE Healthcare). The proteins were eluted by imidazole in buffer A. The buffer was exchanged with phosphate-buffered saline (PBS) by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80°C until use. Protein was quantified by using a BCA protein assay kit (Pierce Chemical, Rockford, IL) with BSA as a standard.

In Vitro Cytotoxic Analysis. In the cytotoxic assay, B16 and CL4-B16 cells were seeded onto a 96-well culture dish at 1×10^4 cells per well. After 24 h, the cells were treated with C-CPE-PSIF for 24 h at the indicated concentrations. The cytotoxicity was determined by a WST-8 kit, according to the manufacturer's instructions (Nacalai Tesque, Kyoto, Japan).

In Vivo Antitumor Activity. Female BALB/c mice (7–8 weeks old) and C57/BL6 mice (7–8 weeks old) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). The mice were housed in an environmentally controlled room at 23 ± 1.5°C with a 12-h light/dark cycle. The animal experiments were performed according to the guidelines of Osaka University.

For an experimental metastasis model, B16 or CL4-B16 cells (5×10^5 or 1×10^6 cells) in 100 μl of PBS were injected intravenously into the tails of C57/BL6 mice. Then, mice received intravenously administered PBS or C-CPE-PSIF three times per week. Two weeks after the inoculation of the cells, the mice were sacrificed, and the number of lung metastasis colonies was counted under a microscope. For antitumor activity in B16 cells, 1×10^5 B16 or CL4-B16 cells were injected subcutaneously into the right flanks of C57BL/6 mice. Vehicle or C-CPE-PSIF was intravenously administered three times per week. Calipers were used to measure the minimal and maximal tumor diameters, and the tumor volume was calculated as $a \times b \times b/2$, where a represents the minimal tumor diameter, and b represents the maximal tumor diameter. The body weights of mice were also monitored.

For antitumor activity in 4T1 cells, 4T1 cells (1×10^5cells) in 50 μl of PBS were injected subcutaneously into the right flanks of BALB/c mice. PBS, C-CPE-PSIF, or adriamycin (ADR) was intravenously administered, and the tumor size and body weight of mice were monitored. Mice were sacrificed 35 days after tumor inoculation, and lung metastasis was evaluated by staining with India ink.

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

Results

Preparation of Claudin-4-Expressing B16 BL6 Cells. To investigate the effects of C-CPE-PSIF on tumor metastasis, we selected murine B16 cells, which have a high propensity

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

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

Suppression of the Primary Tumors and Metastasis of 4T1 Cells. To clarify the potency of a claudin-4-targeting strategy, we investigated the effect of C-CPE-PSIF on a spontaneous metastasis model. 4T1 cells are murine cancer cells that spontaneously metastasize to the lung after being subcutaneously inoculated (Wong et al., 2002). We investigated whether C-CPE-PSIF suppresses the spontaneous lung metastasis of 4T1 cells. On day 33, the tumor volume was 1801.2 ± 848.5 mm^3 in the vehicle-treated group and 740.5 ± 94.6 mm^3 in the group treated with 5 μg/kg C-CPE-PSIF (Fig. 3A). The number of lung metastasis colonies was decreased to 2 ± 1 colonies at 5 μg/kg C-CPE-PSIF (Fig. 3B). A dose of 2 μg/kg C-CPE-PSIF did not suppress tumor growth

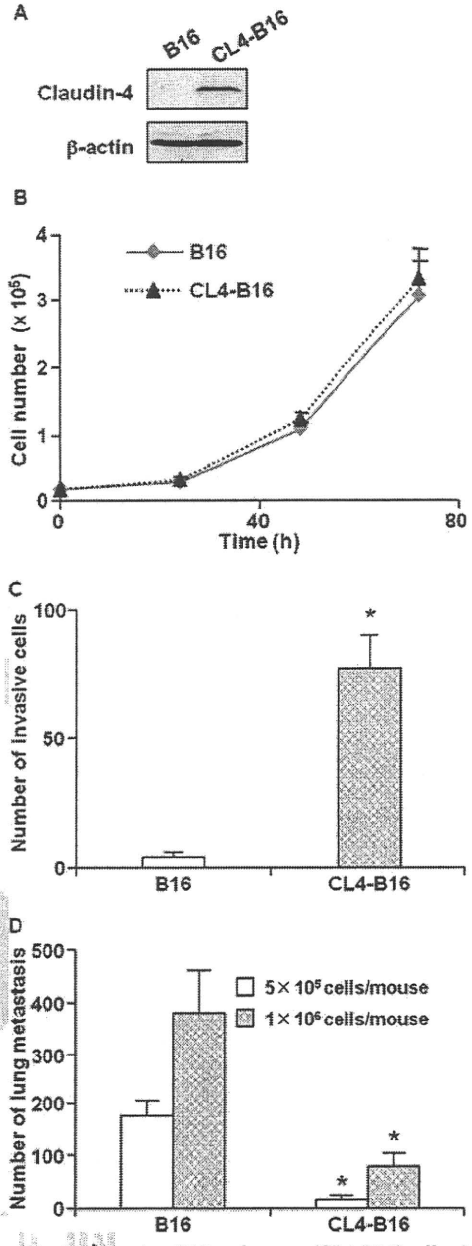

Fig. 1. Claudin-4-expressing B16 melanoma (CL4-B16) cells. A, preparation of CL4-B16 cells. Cell lysates from B16 and CL4-B16 cells were subjected to SDS-polyacrylamide gel electrophoresis, followed by Western blotting with claudin-4 and β-actin. β-Actin is a control for an endogenous protein. B, the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

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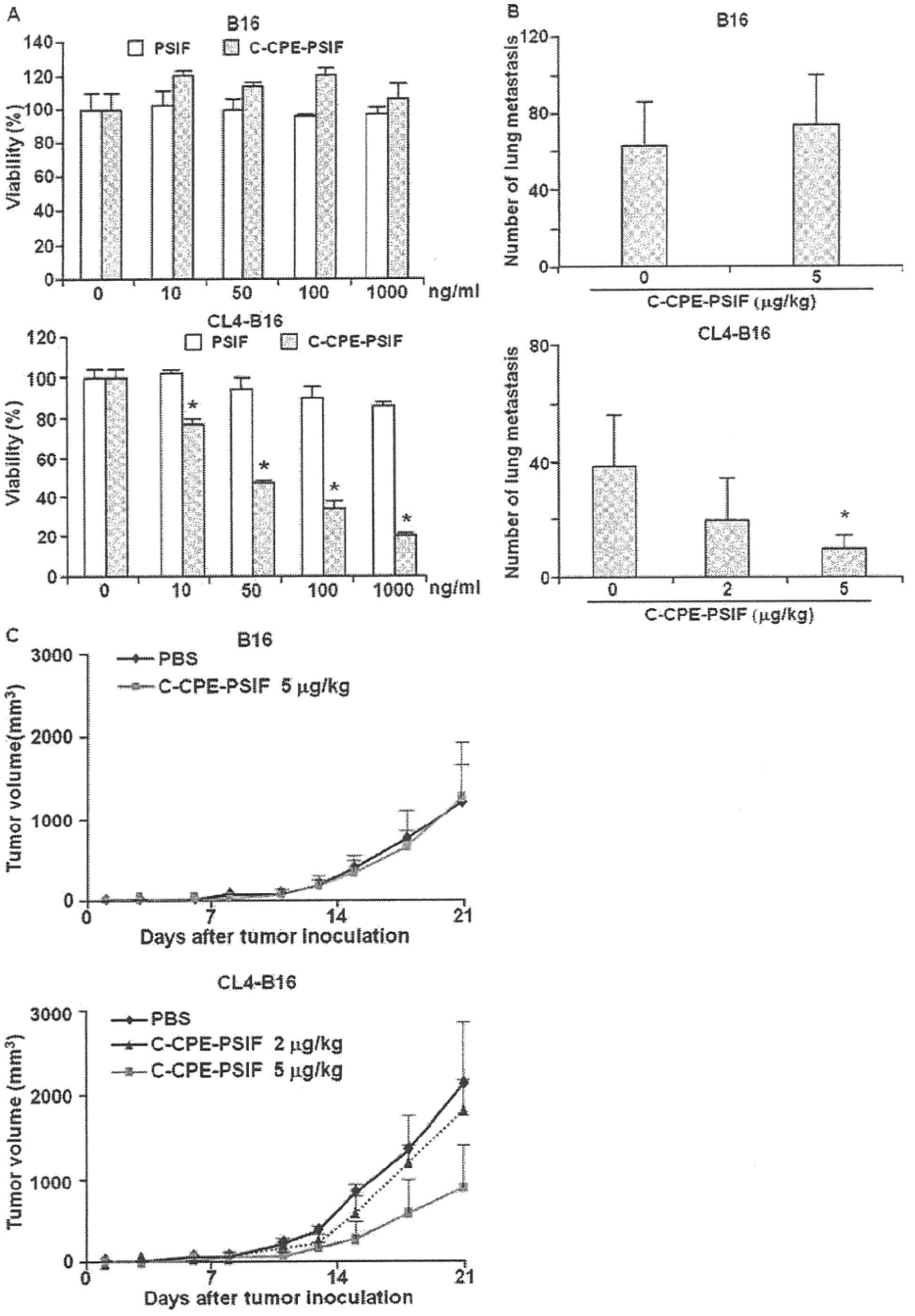

Fig. 2. Antitumor activity of C-CPE-PSIF for CL4-B16 cells. A, cytotoxicity of C-CPE-PSIF in CL4-B16 cells. B16 cells (top) and CL4-B16 cells (bottom) were treated with PSIF or C-CPE-PSIF at the indicated concentrations for 24 h. Cell viability (%) was measured by a WST-8 kit, according to the manufacturer's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

but did prevent lung metastasis. The circulating tumor cells might be more sensitive to C-CPE-PSIF than tumor cells in the solid tumor tissue. C-CPE-PSIF treatments did not cause a decrease in body weight (Fig. 3C), and there were no apparent biochemical side effects (Supplemental Fig. 1). ADR, which is frequently used in clinical chemotherapy, suppressed the tumor growth from 970.3 ± 278.4 to 458.6 ± 51.4 mm^3 at 4 mg/kg (Fig. 3D). As shown in Fig. 3E, 4 mg/kg ADR decreased the number of lung metastasis colonies (24 ± 13 colonies in the vehicle-treated group; 6 ± 4 colonies in the ADR-treated group). However, the ADR-treated mice experienced a 26% loss of body weight, which is a sign of side effects (Fig. 3F). Thus, the antitumor activity of C-CPE-PSIF might be more potent than that of ADR. These results indicate that claudin-4-targeting therapy might be a potent strategy for tumor therapy with a low level of side effects and a high level of antitumor activity.

Discussion

Most malignant tumors are derived from the epithelium, and metastasis is the major cause of death from cancers. In the present study, we found that systemic administration of a claudin-targeting molecule suppressed cancer metastasis, indicating that claudin targeting might be an effective therapy against cancer metastasis.

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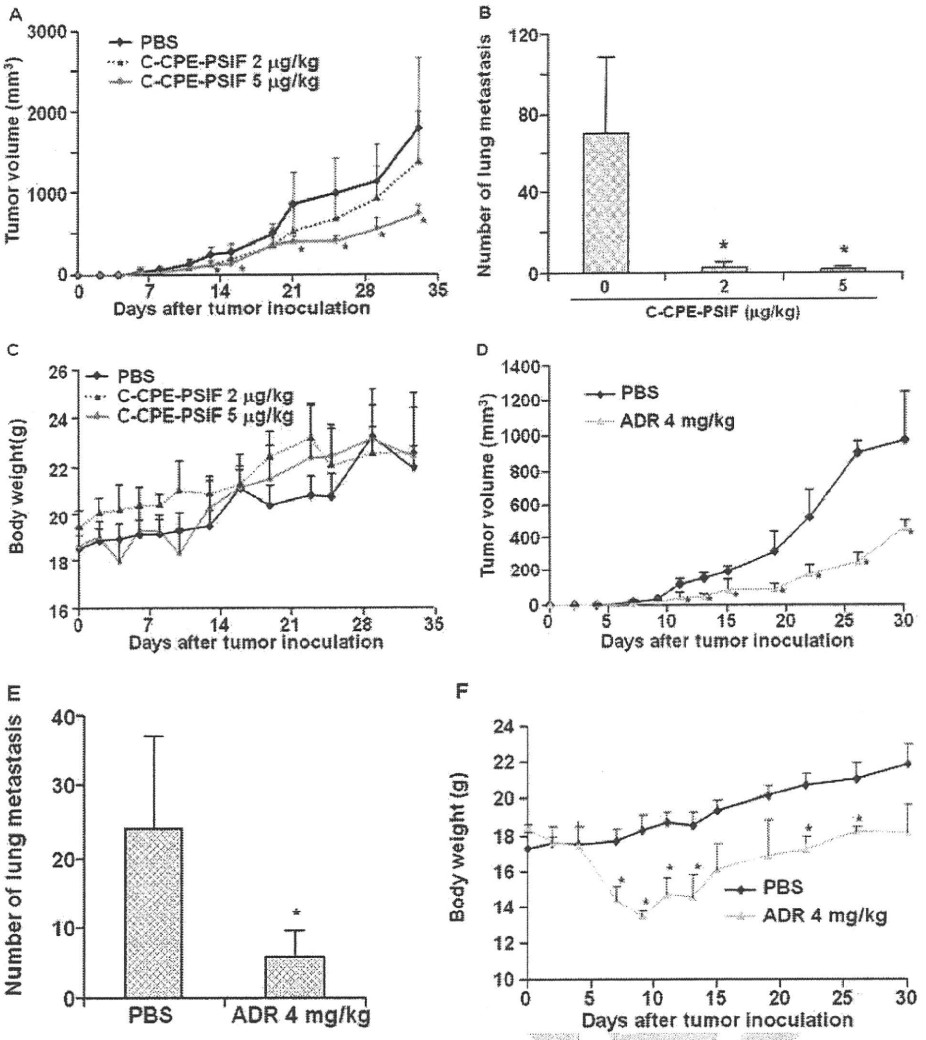

Fig. 3. Antitumor activity of C-CPE-PSIF for murine breast cancer 4T1 cells. 4T1 cells (1×10^5 cells) were intradermally inoculated into the right flanks of mice on day 0, and C-CPE-PSIF (A–C) or ADR (D–F) was intravenously injected three or two times a week at the indicated doses, respectively. Tumor volume (A and D) and body weight (B and E) were monitored. On day 35, the mice were sacrificed, their lungs were stained with India ink, and the number of spontaneous metastases was determined (C and F). Data are shown as means ± S.D. ($n = 5$). *, significantly different from the vehicle-treated group ($p < 0.01$). The data are representative of two independent experiments.

Although the in vitro metastasis activity of CL4-B16 cells was higher than that of parental B16 cells, the in vivo lung metastasis of CL4-B16 cells was lower than that of B16 cells. As shown in Supplemental Fig. 2, claudin-4 increased the invasiveness and migration activity of B16 cells in vitro and decreased lung metastasis in vivo. A possible explanation for this discrepancy might be the experimental model, which evaluates a different stage of metastasis. The migration and invasion activity involved in the early stage of metastasis was investigated in the in vitro analysis, whereas extravasation and colonization to an organ involved in the late stage of metastasis was evaluated by the in vivo experiment. The altered expression of claudin-4 changed the metastasis of 4T1 cells to the heart and liver, suggesting that claudin affects organ-specific metastasis (Erin et al., 2009). Claudin-4 might suppress the lung metastasis of B16 cells.

Claudin is a structural and functional component of TJs (Furuse and Tsukita, 2006). What is the role of claudin in metastasis? Metastasis is composed of three steps: leaving the primary site, entering the blood flow, and invading the distant site. In the first step, the combination of claudin members in the TJ strands may be important. The claudin family contains at least 24 members. Claudin is believed to form homo- and hetero-type claudin strands, and the pattern of the strands differs among tissues and determines the properties of TJ seals (Furuse and Tsukita, 2006). For example, rigid TJ seals were formed when claudin-11 or claudin-15 was exogenously expressed in Madin-Darby canine kidney cells, whereas the expression of claudin-11 or claudin-15 reduced the TJ integrity in LLC-PK1 cells by its dominant negative effects on TJ sealing (Van Itallie et al., 2003). Dominant negative effects of claudin-4 on the TJ barrier might contribute to an acceleration in the detachment of cancer cells from the primary tumor tissue. In the second and third steps of metastasis, cancer cells must move through the extracellular matrix at the primary site and the distant site. Cancer cells must degrade the extracellular matrix by the expression of matrix metalloproteinase and increase their motility. Claudin expression enhanced invasion with increased matrix metalloproteinase activity (Agarwal et al., 2005). There is a relationship between the levels of claudin-1/claudin-4 and the metastasis of human cancers, including hepatic, colonic, ovarian, and gastric cancers (Miwa et al., 2001; Agarwal et al., 2005; Resnick et al., 2005; Halder et al., 2008; Lee et al., 2008). The overexpression of claudin suppressed cancer metastasis in human pancreatic and gastric cancers (Michl et al., 2003; Mima et al., 2005; Ohtani et al., 2009). Claudin-4 suppressed or accelerated in vitro and in

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vivo metastasis of human cancer cells (Agarwal et al., 2005; Ohtani et al., 2009). Cell–cell interaction through TJs regulates cell growth signaling (Matter et al., 2005). Taken together, these findings indicate that claudin family members might control several steps of cancer metastasis. The precise molecular mechanism and role of claudin in cancer metastasis remain to be determined.

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

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It is difficult to prepare recombinant claudin protein because of its hydrophobic property, and claudin has low antigenicity. Until recently, an antibody against the extracellular loop domain of claudin had never been successfully prepared, and C-CPE was the only known claudin binder. Recently, Romani et al. (2009) prepared a single-chain antibody fragment against claudin-3 by using phage display technology. They found that the antibody fragment binds to ovarian and uterine carcinoma cells in vitro. More importantly, a therapeutic monoclonal antibody against claudin-4 was developed. Suzuki et al. (2009) successfully prepared anticlaudin-4 antibody by immunizing claudin-4-expressing tumor cells into a mouse with autoimmune disease. The antibody mediates antibody-dependent cellular cytotoxicity and both in vitro and in vivo antitumor activity. Although the preparation of anticlaudin antibody may lead to a breakthrough in cancer therapy, the immunogenicity associated with immunotoxin clinical therapies is a future problem (Kreitman and Pastan, 2006). The C-terminal 30 amino acids are the minimum functional domain of C-CPE to bind to claudin-4 (Hanna et al., 1991). The C-terminal 30-amino-acid fragment was used to deliver a cytokine to claudin-4-expressing cells by genetic fusion (Yuan et al., 2009). Humanized antibody and the claudin-4-targeting peptide may be useful for cancer therapy in the near future.

In summary, this is the first report to indicate that systemic injection of a claudin-targeting molecule suppresses tumor growth and metastasis. Hematologic cells do not develop TJs; therefore, a claudin-targeting therapy may have no hematologic toxicity. We anticipate that claudin targeting will be a potent strategy for cancer therapy.

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A Novel Screening System for Claudin Binder Using Baculoviral Display

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Abstract

Recent progress in cell biology has provided new insight into the claudin (CL) family of integral membrane proteins, which contains more than 20 members, as a target for pharmaceutical therapy. Few ligands for CL have been identified because it is difficult to prepare CL in an intact form. In the present study, we developed a method to screen for CL binders by using the budded baculovirus (BV) display system. CL4-displaying BV interacted with a CL4 binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), but it did not interact with C-CPE that was mutated in its CL4-binding region. C-CPE did not interact with BV and CL1-displaying BV. We used CL4-displaying BV to select CL4-binding phage in a mixture of a scFv-phage and C-CPE-phage. The percentage of C-CPE-phage in the phage mixture increased from 16.7% before selection to 92% after selection, indicating that CL-displaying BV may be useful for the selection of CL binders. We prepared a C-CPE phage library by mutating the functional amino acids. We screened the library for CL4 binders by affinity to CL4-displaying BV, and we found that the novel CL4 binders modulated the tight-junction barrier. These findings indicate that the CL-displaying BV system may be a promising method to produce a novel CL binder and modulator.

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Introduction

Tight junctions (TJ) are intercellular adhesion complexes in epithelial and endothelial cells; TJs are located in the most apical part of the complexes [1]. TJs have a barrier function and a fence function [2–4]. TJs contribute to epithelial and endothelial barrier functions by restricting the diffusion of solutes through the paracellular pathway. TJs maintain cellular polarity by preventing the free movement of membrane proteins between the apical and basal membranes [5]. Loss of cell-cell adhesion and cellular polarity commonly occurs in the early stages of cancer [6]. Modulation of the TJ barrier function can be a method to enhance drug absorption, and TJ components exposed on the surface of cancer cells can be a target for cancer therapy.

Biochemical analyses of TJs have identified TJ components, such as occludin, claudins (CLs) and junction adhesion molecule [7]. The CL family contains more than 20 integral tetra-transmembrane proteins that play pivotal roles in the TJ barrier and fence functions. CL1-deficient mice lack the epidermal barrier, while CL5-deficient mice lack the blood-brain barrier [8,9], indicating that the regulation of the TJ barrier by modulation of CLs may be a promising method for drug delivery. *Clostridium perfringens* enterotoxin (CPE) causes food poisoning in humans [10]. An interaction between the C-terminal domain of CPE (C-CPE) with CL4 deregulates the TJ barrier [11,12]. We previously found that C-CPE enhances jejunal absorption through its interaction with CL4, indicating that a CL binder is a potent drug-delivery system [13].

The majority of lethal cancers are derived from epithelial tissues [14]. Malignant tumor cells frequently exhibit abnormal TJ function, followed by the deregulation of cellular polarity and intercellular contact, which is commonly observed in both advanced tumors and the early stages of carcinogenesis [6]. Some CLs are overexpressed in various types of cancers. For example, CL3 and CL4 are overexpressed in breast, prostate, ovarian, pancreatic and gastric cancers. CL1, CL7, CL10 and CL16 are overexpressed in colon, gastric, thyroid and ovarian cancers, respectively [15,16]. These findings indicate that the CLs may be a target molecule for cancer therapy. A receptor for CPE is CL4 [11,12]. CPE has anti-tumor activity against human pancreatic and ovarian cancers without side effects [17,18]. The CLs binders will be useful for cancer-targeting therapy.

As above, recent investigations of CLs provide new insight into their use as pharmaceutical agents; for example, a CL binder may be used in drug delivery and anti-tumor therapy. Selection of a CL binder by using a recombinant CL protein is a putative method to

prepare a CL binder. However, CLs are four-transmembrane proteins with high hydrophobicity; there has been little success in the preparation of intact CL protein. Recently, a novel type of protein expression system that uses baculovirus has been developed. Membrane proteins are displayed on the budded baculovirus (BV) in their active form [19–21], indicating that the BV system may be useful for the preparation of a CL binder. In the present study, we investigated whether a CL binder was screened by using a CL-displaying BV.

Results

Preparation of CL4-displaying BV

C-CPE is the only known CL binder and modulator [12,13,22]. C-CPE has affinity to CL4 in a nanomolar range [23]. We chose C-CPE and CL4 as models of the CL binder and CL, respectively. Several reports indicate that membrane proteins expressed on the surface of BV are in an intact form [19–21]. To check the expression of CL4 on the BV, we performed immunoblot analysis of the lysate of CL4-BV against CL4. As shown in Fig. 1A, CL4 was detected in the virus lysates. To determine if the CL4 expressed on the virus has an intact form, we performed enzyme-linked immunosorbent assay (ELISA) with CL4-BV-coated immunoplates. C-CPE binds to the extracellular loop domain of CL4 [23]. After the addition of C-CPE to the CL4-BV-coated plate, the C-CPE bound to the CL4-BV-coated plate was detected by anti-his-tag antibody, followed by incubation with horseradish peroxidase-labeled antibody. C-CPE was dose-dependently bound to CL4-BV, whereas C-CPE did not interact with wild-BV (Fig. 1B). Deletion of the CL4-binding region (C-CPE303) attenuated the interaction of C-CPE with CL4-BV (Fig. 1C). Together, these results indicate that the CL4 displayed on BV may have an intact extracellular loop region.

Selection of C-CPE-phage by using CL4-BV

We next examined the interaction between C-CPE-phage and CL4-BV. As shown in Fig. 2A, C-CPE-phage bound to CL4-BV but not to wild-BV, and a scFv-phage did not bind to CL4-BV. To determine if CL-BV can be used to select CL binders, we prepared a mixture of C-CPE-phage and scFv-phage at a ratio of 2:10 and used CL4-BV to select CL4-binding phage in the mixtures. The amount of C-CPE-phage was increased to 11 of 12 clones in the mixture (Fig. 2B), indicating that CL-BV may be useful in the preparation of CL binders.

We previously found that each substitution of S304, S305, S307, N309, S313 and K318 with alanine increased the binding of C-CPE to CL4 [24]. Here, we prepared a phage library for C-CPE by randomly changing the functional 6 amino acids to any of the 20 amino acids. To confirm the diversity of the library, we checked the sequences of 17 randomly isolated clones. Each of the 17 clones had a different sequence, indicating that the library has a diverse population of C-CPE mutants (Table 1).

Then, we screened the CL4-binding phage by their affinity to CL4-BV. After addition of the C-CPE library to CL4-BV-adsorbed tubes, the CL4-BV-bound phages were recovered (1st screening). We repeated this screening process two more times (2nd screening and 3rd screening). If the number of CL4-bound phage is increased during the screening, the ratio of the incubated phage titers to the recovered phage titers will increase. As shown in Fig. 3A, the ratio was increased during screening from 4.5×10^{-7} to 5.5×10^{-5}, indicating that the screening system for CL4 binders may work. Indeed, the number of monoclonal phage clones with high affinity to CL4-BV was increased after the 3rd screening compared with that after the 2nd screening (Fig. 3B).

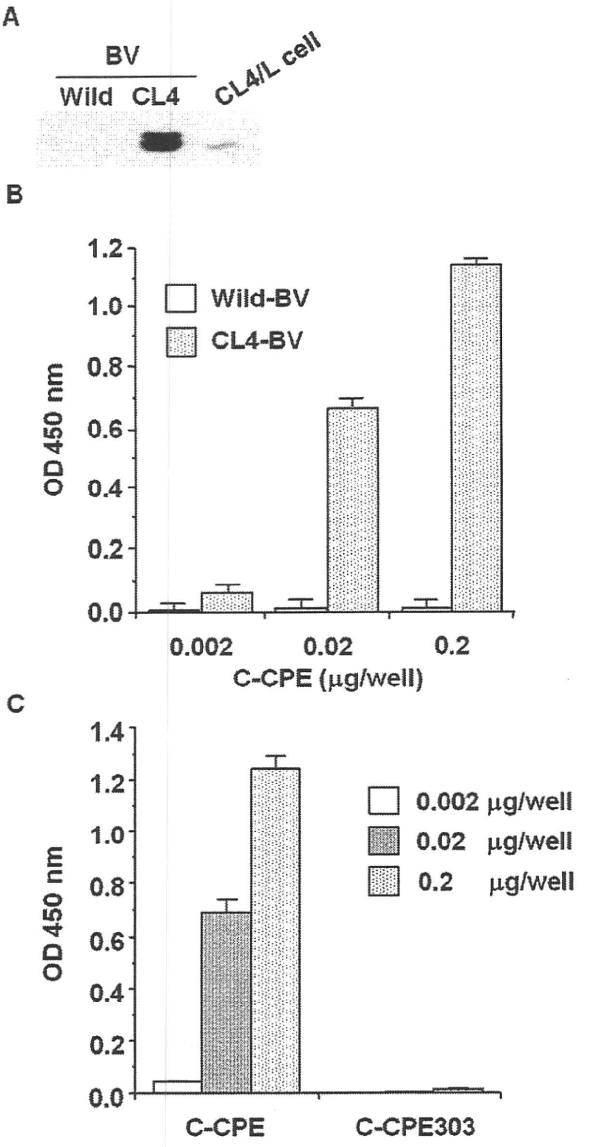

Figure 1. Preparation of CL4-displaying BV. A) Immunoblot analysis. Wild-BV and CL4-BV (0.1 μg/lane) were subjected to SDS-PAGE, followed by immunoblot analysis with anti-CL4 antibody. The lysate of CL4-expressing L (CL4/L) cells was used as a positive control. B, C) Interaction of a CL4 binder with CL4-BV. Immunotubes were coated with the wild-BV or CL4-BV, and C-CPE (B) or mutated C-CPE (C) was added to the BV-coated immunotubes at the indicated concentration. C-CPE bound to the BV-coated tubes was detected by ELISA with an anti-his-tag antibody.
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We analyzed the sequences of the CL4-BV-bound phages and got novel CL4-binder candidates with amino acid sequences that differed from the wild-type sequence (Table 2). To investigate their CL4-binding, we prepared the recombinant proteins of the binders and investigated their interaction with CL4 by ELISA with CL-BVs. As shown in Fig. 4A, the novel C-CPE derivatives had affinity to CL4 but not CL1. Next, we investigated whether the novel CL4 binders modulate TJ barrier in Caco-2 monolayer cell sheets, a popular model for the evaluation of TJ barriers [25].

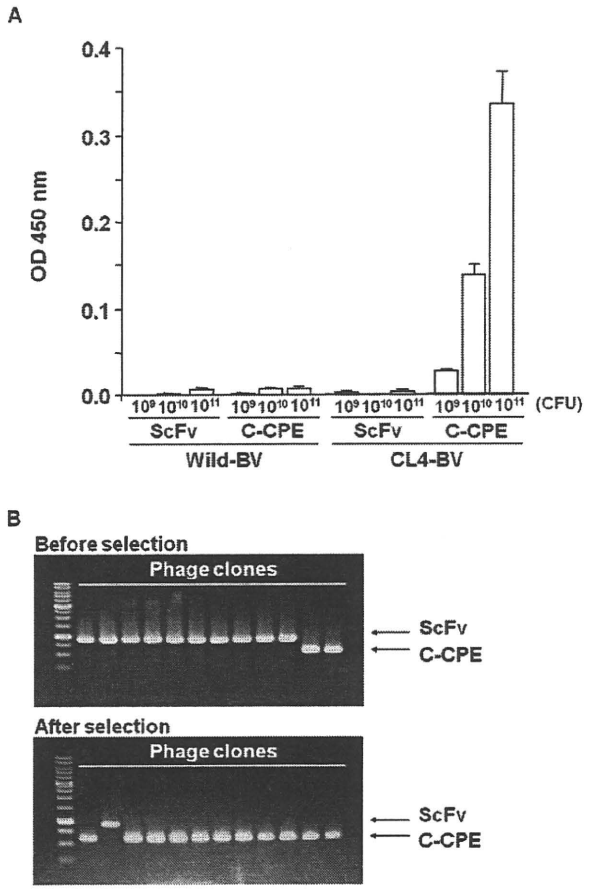

Figure 2. Selection of C-CPE-displaying phage by using the CL4-BV system. A) Interaction of C-CPE-displaying phage with CL4-BV. Wild-BV or CL4-BV was coated on an immunoplate, and then scFv-displaying phage or C-CPE-displaying phage was added to the BV-coated immunoplate at the indicated concentrations. The BV-bound phages were detected by ELISA with anti-M13 antibody as described in Materials and methods. Data are representative of two independent experiments. Data are means ± SD (n = 3). B) Enrichment of C-CPE-displaying phage by the BV system. A mixture of scFv-phage and C-CPE-phage (mixing ratio of scFv-phage to C-CPE-phage = 2:10) was incubated with a CL4-BV-coated immunotube, and the bound phages were recovered. Each phage clone was identified by PCR amplification, followed by agarose gel electrophoresis. Upper and lower pictures are before and after the selection, respectively. The putative sizes of the PCR products are 856 and 523 bp in scFv and C-CPE, respectively. The data are representative of two independent experiments.
doi:10.1371/journal.pone.0016611.g002

Treatment of the cells with C-CPE resulted in decreased transepithelial electrical resistance (TEER) values, a marker of TJ integrity, and the TEER values increased after removal of C-CPE. The C-CPE derivatives (clones 1–5) had TJ-modulating activity similar to that of C-CPE (Fig. 4B).

Discussion

CL is a promising target for pharmaceutical therapy. However, CL has low antigenicity, and there has been little success in the preparation of monoclonal antibody against the extracellular loop region of CL. The three-dimensional structure of CL has never been determined, so it is impossible to perform a theoretical design of a CL binder based on the structural information. In the present study, we developed a novel screening system for CL binders by using a BV system and a C-CPE phage display library, and we used this system to identify novel CL4 binders.

Table 1. C-CPE phage library.

	304	305	307	309	313	318
C-CPE	**S**	**S**	**S**	**N**	**S**	**K**
Clone 1	V	T	C	V	N	K
2	C	P	A	H	L	T
3	A	G	G	V	P	P
4	R	G	H	L	E	H
5	A	A	P	S	R	Q
6	P	A	P	D	P	A
7	C	T	T	T	N	K
8	H	P	S	P	G	H
9	R	G	G	R	N	R
10	A	P	S	T	Q	P
11	V	L	G	N	M	R
12	P	P	A	T	F	R
13	G	D	C	S	N	L
14	F	R	V	F	R	N
15	S	Q	Q	W	T	T
16	S	R	L	E	W	Q
17	K	R	E	R	Q	S

Phage clones were randomly picked up from the C-CPE phage library, and the amino acids sequences of C-CPE mutant were analyzed.
doi:10.1371/journal.pone.0016611.t001

In ligand screening, the preparation of a receptor for the ligand is very critical. Membrane proteins are especially difficult to prepare as recombinant protein with an intact structure. Functional membrane proteins such as cell-surface proteins are heterologously expressed on BV in their native forms [19–21]. Interactions between membrane proteins can be detected by using receptor-displaying and ligand-displaying BV [21]. In the present report, we found that CL4-BV interacts with a CL4 binder, C-CPE, but it does not interact with C-CPE303 that lacks the CL4-binding residues of C-CPE. The CL4-binding site of C-CPE corresponds to that of CPE; so, the second extracellular loop of CL appears to be the C-CPE-binding site [23,26]. These findings indicate that CL4 displayed on BV may have native form. We anticipate that CL-BV will be useful for the preparation of CL binders, such as peptides and antibodies.

To the best of our knowledge, the preparation of CL binder has been performed by only four groups. Offner et al. prepared polyclonal antibodies against extracellular domains of CL3 and CL4 [27], Ling et al. screened peptide types of CL4 binder by using a 12-mer peptide phage display library and CL4-expressing cells [28], Suzuki et al. generated a monoclonal antibody against the second extracellular loop of CL4 from mice immunized with a human pancreatic cancer cell line [29] and Romani et al. screened scFv against CL3 by using a human antibody phage display library [30]. However, the CL modulators have never been developed; thus, C-CPE is the only known CL4 modulator [12]. In the present study, we prepared a C-CPE phage library containing C-CPE mutants in which each of the 6 functional amino acids was randomly replaced with an amino acid, and we isolated CL4 binders by using CL4-BV as a screening ligand. Interestingly, all of

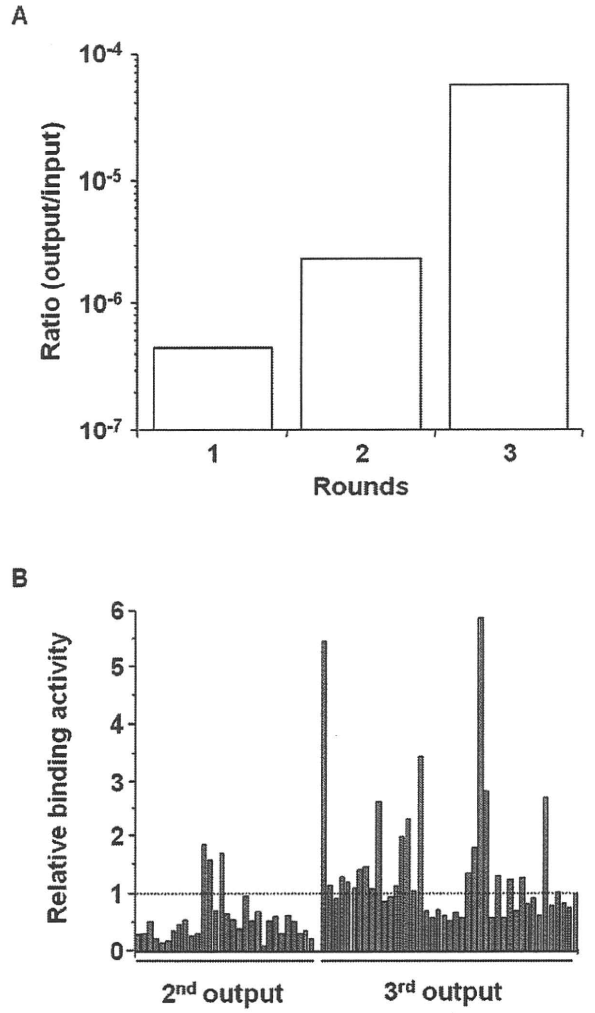

Figure 3. Screening of a novel CL4 binder. A) Enrichment of phages with affinity to CL4-BV. CL4-BVs coated on immunotubes were incubated with the C-CPE-derivative phage library at 1.6×10^{12} CFU titer (1^{st} input phage). The phages bound to CL4-BV were recovered (1^{st} output phage). The CL4-BV-binding phages were subjected to two additional cycles of the incubation and wash step, resulting in 2^{nd}, 3^{rd} output phage. The ratio of output phage to input phage titers was calculated. B) Monoclonal analysis of C-CPE-derivative phage. CL4-BV-bound phage clones were isolated from the 2^{nd} and 3^{rd} output phages, and the interaction of the monoclonal phage with CL4-BV was examined by ELISA with anti-M13 antibody as described in Materials and methods. Data are expressed as relative binding to that of C-CPE-phage indicated by the most right column.
doi:10.1371/journal.pone.0016611.g003

the CL4 binders modulated TJ barriers. We are investigating why the substitution of the amino acids with the other amino acids modulated CL4. These findings indicate that a BV screening system with a C-CPE library may be a powerful method to develop CL modulators.

The CL family forms various types of TJ barriers through combinations of its more than 20 members in homophilic/heterophilic CL strands [31,32]. Intercellular proteins ZO-1 and ZO-2 determine the localization of CL strands [33]. If a screening system to reconstitute heterogeneous CL strands with ZO-1 and/or ZO-2 is developed, then useful and effective CL modulators can be identified. In this point, the BV system has extremely superior features. G protein and G protein-coupled receptors have been

Table 2. CL4-binding phages.

	304	305	307	309	313	318
C-CPE	S	S	S	N	S	K
Clone 1	R	V	S	A	R	R
2	R	S	V	A	R	K
3	G	D	G	R	T	R
4	S	A	P	R	S	A
5	R	S	L	K	S	K

The sequences of C-CPE mutant in the CL4-binding phages were analyzed.
doi:10.1371/journal.pone.0016611.t002

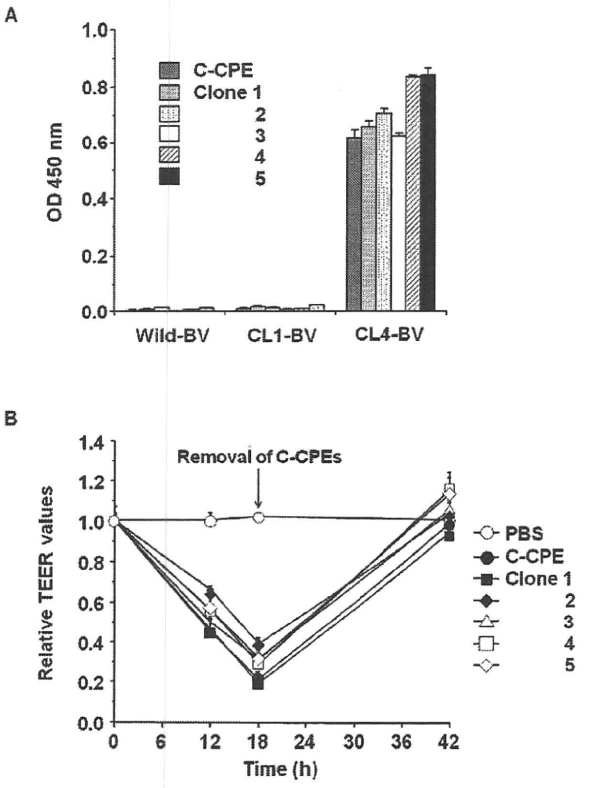

Figure 4. Isolation of a novel CL4 modulator. A) Interaction of the C-CPE derivatives with CL4. C-CPE derivatives were prepared as his-tagged recombinant proteins. The C-CPE derivatives (0.02 μg) were added to CL-BV-coated immunoplates, followed by detection of the C-CPE derivatives bound to CL-BV. Data are means ± SD (n = 4). B) Modulation of tight junction-barriers. Caco-2 cells were cultured on Transwell™ chambers. When TEER values reach a plateau, the cells were treated with C-CPE or C-CPE derivatives at the indicated concentrations. After 18 h of exposure to the C-CPEs, the cells were washed with medium to remove C-CPEs, and then the cells were cultured for an additional 24 h. Changes in TEER values were monitored during the C-CPEs treatment. Relative TEER values were calculated as the ratio of TEER values at 0 h. Data are representative of two independent experiments. The data are means ± SD (n = 4).
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functionally reconstituted in BV [20,34], and functional γ-secretase complexes have also been reconstituted on BV [35]. In the near future, the reconstituted CL system on BV will be developed and used for the screening of CL binders and modulators, hopefully leading to breakthroughs in pharmaceutical therapies that target CLs.

Materials and Methods

Recombinant BV construction and Sf9 cell culture

Recombinant BV was prepared by using the Bac-to-Bac expression system, according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD). Briefly, mouse CL1 and CL4 cDNA (kind gifts from Dr. M Furuse, Kobe University, Japan) were inserted into pFastBac1, and the resulting plasmids were transduced into DH10Bac *E. Coli* cells. Recombinant bacmid DNA was extracted from the cells. Sf9 cells were transduced with the bacmid coding CL, and the recombinant BV was recovered by centrifugation of the conditioned medium [36].

Preparation of the BV fractions

Sf9 cells (2×10^6 cells) were infected with recombinant BV at a multiplicity of infection of 5. Seventy-two hours after infection, the BV fraction was recovered from the culture supernatant of infected Sf9 cells by centrifugation. The pellets of the BV fraction were resuspended in Tris-buffered saline (TBS) containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and then stored at 4°C until use. The expression of CL1 and CL4 in the BV was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis with anti-CL antibodies (Zymed Laboratory, South San Francisco, CA).

Preparation of mutant C-CPE library

C-CPE fragments in which the functional amino acids (S304, S305, S307, N309, S313 and K318) [24] were randomly mutated were prepared by polymerase chain reaction (PCR) with pET-H_{10}PER as a template, a forward primer (5′-catgccatggccgatatagaaaaagaaatccttgatttagctgctg-3′, Nco I site is underlined) and a reverse primer (5′-ttttccttttgcggccgcaaa*nnn*ttgaaataatat*nnn*ataagggta*nnn*tcc*nnn*ata*nnnnn*attagcttt-3′, Not I site is underlined, and the randomly mutated amino acids are in italics). The PCR fragments were inserted into a pY03 phagemid at the NcoI/NotI sites [22]. The resultant phagemid containing the C-CPE mutant library was transduced into *E. coli* TG1 cells, and then the cells were stored at −80°C.

Preparation of phage

TG1 cells containing phagemid coding a scFv, C-CPE, C-CPE mutant or C-CPE mutant library were culture in 2YT medium containing 2% glucose and ampicillin. When the cells grew to a growing phage, M13K07 helper phages (Invitrogen) were added, and the medium was changed into 2YT medium containing ampicillin and kanamycin. After an additional 6 h of culture, the phages in the conditioned medium were precipitated with polyethylene glycol. The phages were suspended in phosphate-buffered saline (PBS) and stored at 4°C until use.

ELISA

Wild-BVs or CL-BVs (0.5 μg/well) were adsorbed onto an immunoplate (Greiner Bio-One, Frickenhausen, Germany). The wells were washed with PBS and blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). C-CPEs or phages were incubated in the immunoplate, and the BV-bound C-CPEs or phages were detected by using anti-his-tag antibody (Novagen, Darmstadt, Germany) or anti-M13 antibody (Amersham-Pharmacia Biotech, Uppsala, Sweden), respectively, horseradish peroxidase-labelled secondary antibody and TMB peroxidase substrate (Nacalai Tesque, Kyoto, Japan). The immunoreactive C-CPEs or phages were quantified by the measurement of absorbance at 450 nm. In the screening of phages, the data were normalized by the amounts of phages, which were quantified by ELISA for the FLAG-tag contained in the coat protein.

Selection of phage by using BV

A total of 0.5 μg of BV was adsorbed onto an immunotube (Nunc, Roskilde, Denmark). The tube was washed with PBS and blocked with TBS containing 4.0% BlockAce. The BV-coated tubes were incubated with mixture of phages, and then the tubes were washed 15 times with PBS and 15 times with PBS containing 0.05% Tween 20. The phages bound to the tube were eluted with 100 mM HCl. TG1 cells were infected with the eluted phages, and phages were prepared as described above. The resulting phages were subjected to repeated selection by using the BV-coated immunotubes.

Identification of a phage clone

To identify an isolated phage clone, we performed PCR or sequencing analysis. We amplified the inserted fragment into the phagemid by PCR using forward primer 5′-caggaaacagctatgac-3′ and reverse primer 5′-gtaaatgaattttctgtatgagg-3′. The resultant PCR products were subjected to agarose gel electrophoresis followed by staining with ethidium bromide. We performed a sequence analysis with primer 5′-gtaaatgaattttctgtatgagg-3′.

Measurement of phage titer

To quantify the concentration of phages, we measured the titer (colony formation unit (CFU)/ml) of the phage solution. Briefly, the phage solution was diluted to 10^{-5}–10^{-10} with PBS. The diluted solution was seeded onto Petrifilm™ (Tech-Jam, Osaka, Japan). After 24 h of incubation, the colonies were counted, and the titer was calculated.

Purification of C-CPE mutants

C-CPE and C-CPE303, in which the CL-4 binding region of C-CPE was deleted, were prepared as described previously [13]. To prepare plasmid containing C-CPE mutants, the C-CPE mutant fragment was PCR-amplified by using phagemids coding C-CPE mutants as a template. The resulting PCR fragment was inserted into pET16b, and the sequence was confirmed. The plasmids were transduced into *E. coli* strain BL21 (DE3), and production of mutant 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 phenylmethanesulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol) that was supplemented with 8 M urea when necessary. The lysates were applied to HiTrap™ Chelating HP (GE Healthcare, Buckinghamshire, UK), and mutant C-CPEs were eluted with buffer A containing 100–400 mM imidazole. The buffer was exchanged with PBS by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80°C until use. Purification of the mutant C-CPEs was confirmed by SDS-PAGE, followed by staining with Coomassie Brilliant Blue and by immunoblotting with anti-his-tag antibody (Novagen). Protein was quantified by using a BCA protein assay kit with bovine serum albumin as a standard (Pierce Chemical, Rockford, IL).