

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 μ g 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 \pm 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 immunocompetent cells by FAE is a trigger of mucosal immune responses

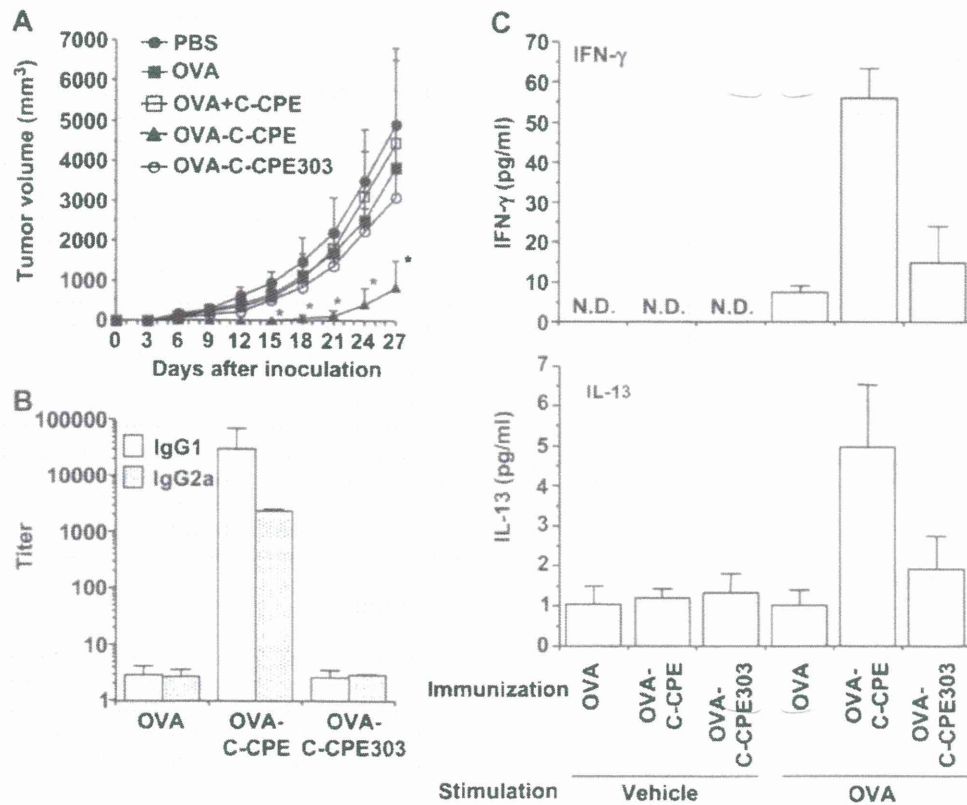


Fig. 6. Anti-tumor activity induced by immunization with OVA-C-CPE in an EG7 cancer model. A) Protective immune response against tumor challenge. C57BL/6 mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA-C-CPE, or OVA-C-CPE303 (5 μ g of OVA) once a week for 3 weeks. Seven 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 \pm 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 μ g 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 \pm SD ($n = 4$). N.D., not detected. The results are representative of two independent experiments.

[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 (data not shown). 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 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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肝細胞機能制御を目的とした新規培養システムの開発

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Development of Novel Culture System for Regulation of Hepatocyte Function

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Cultured hepatocytes are expected to be used for drug screening and bioartificial liver. Since hepatocytes lose their functions very rapidly *in vitro*, many attempts have been made to maintain their viability and functions. First, we want to introduce the surface modification of culture substrate using a starburst dendrimer. Addition of fructose to the terminal of the dendrimer was shown to be effective in maintaining hepatocyte function. As the second topic, we will show results of the use of a three-dimensional carrier for hepatocyte cultivation. Hepatocytes and bone marrow stromal cells were cocultured in silane beads, and packed into a radial flow-type bioreactor. The perfusion culture showed the effectiveness of bone marrow stromal cells for the maintenance of hepatocyte function. The next topic will be the trial of adenoviral gene transfer into hepatocytes. Thioredoxin gene was chosen because the products play important roles in redox control and antiapoptosis. The introduction of the gene could inhibit apoptosis and maintain the hepatocyte viability. Finally, we want to introduce the results on differentiation of stem cells into hepatocytes, because it is very difficult to obtain sufficient number of human hepatocytes. Human mesenchymal stem cells were cultured in the presence of several protein factors and the hepatocyte-specific marker was expressed after 2 weeks of induction culture. The use of human stem cells could be an important strategy for the support of a drug development system.

Key words—dendrimer; hepatocyte; radial flow-type bioreactor; mesenchymal stem cell

1. はじめに

培養肝細胞は医薬品開発のスクリーニング系やバイオ人工肝臓への応用が期待されているがその機能は *viability* の低下に伴い急速に消失していくため、機能維持を目的とした研究が活発に行われている。本総説ではこれまで我々が検討してきた培養基材の表面修飾、3次元培養、遺伝子導入、肝細胞の分化誘導について紹介する。足場依存性の細胞は培養基材の性質によって *viability* が著しく変動することが知られており、機能維持の成否は優れた培養基材の開発に依存していると言っても過言ではない。そこで機能性材料として注目されている樹木状

高分子デンドリマーを用いた基材表面修飾の検討において、デンドリマーの末端にフルクトースを付加すると培養肝細胞の生存性、機能が良好に維持されたことを示す。また培養用ディッシュを用いた2次元培養では応用範囲が限られるため培養工学的検討として多孔性担体を用いた3次元培養の試みを紹介する。さらに細胞を接着させた3次元担体をラジアルフロー型バイオリアクターに充填し灌流培養を行った検討において、肝細胞と骨髄間質細胞との共培養が機能維持に効果的であったことを示す。次に遺伝子工学的アプローチとしてアデノウィルスベクターを用いてチオレドキシンの遺伝子を導入することにより、肝細胞のアポトーシスを制御することが可能であったことを紹介する。最後に、細胞源に関する検討として再生医工学的アプローチによりヒト由来間葉系幹細胞を肝細胞へ誘導する試みを紹介し創薬支援システムへの応用について考察したい。

2. 機能性培養基材による肝機能制御

1985年に Tomalia らによって報告されたデンド

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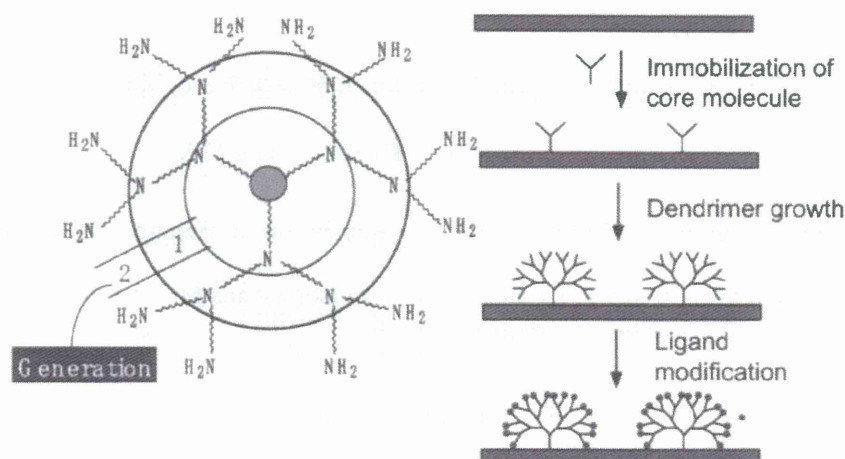


Fig. 1. Structure of Polyamidoamine Dendrimer and Application for Cell Culture

リマーは樹木状多分岐高分子であり、¹⁾様々な領域でその応用が検討されている。直径は約数十 nm の分子であり、Fig. 1 で示すように、中心部分のコア、骨格分子、末端アミノ基から構成され、正確な分子設計が可能である。デンドリマーは規則的な枝分かれ構造を有する分子で、中心のコア分子から、段階的に伸長反応を行うことで枝分かれ数を増加させることができる。また、伸長反応を繰り返すことにより最外部の密度が高くなり、内部の密度が低くなっている。Figure 1 に示すようなポリアミドアミンデンドリマーは末端アミノ基部分の外表面が正に帯電しており、反応性が高い特徴を有している。このような特徴を活かして、様々な分野でデンドリマーの研究が行われている。²⁻⁵⁾ 医療分野へのデンドリマーの応用としては、内部の密度が粗であることを利用して、デンドリマー内部に薬物や遺伝子の封入、また外部の反応性の高さを利用して外部固定を行っている。デンドリマーにアンチセンス遺伝子を導入、またデンドリマー外部に薬物を固定化することによる薬物の徐放化の検討も行われている。

このようなデンドリマーの特徴を活かし、筆者らはデンドリマーにリガンド分子を結合させ、肝細胞培養基材とする方法を考案した。Figure 2 に示すようにカリウム *tert*-ブトキシドを用い基材表面にヒドロキシル基を導入し、グルタルアルデヒドを介してデンドリマーの固定化を行った。世代増加反応はこの反応を繰り返すことにより行い、最後に末端アミノ基へリガンド分子を結合させた。

筆者らはこれまでにキトサンゲル上で肝実質細胞

を培養することに成功しており、特にキトサンの分子内アミノ基をフルクトースにより修飾したフルクトースキトサン上では未修飾のキトサンゲルよりも多くの細胞が接着し、肝特異的機能を維持することを報告した。そこでフルクトースに注目し、フルクトースをリガンドとしたフルクトースデンドリマーについて検討した。細胞非接着性のポリスチレンプレートにデンドリマーを固定化し、リガンドとしてフルクトースを修飾した。その結果、デンドリマーの世代数増加に伴い、修飾されたフルクトースも増加することを確認した。フルクトースデンドリマー上で数日培養を行うと成長因子などの添加なしに、細胞が高機能化されると言われるスフェロイド（球状組織体）を形成した。⁶⁾ しかし、ここではスフェロイドの接着性が弱く、さらに接着性を上げる必要が生じた。そこで、リガンドとしてフルクトースと、肝細胞表面に存在するアシアロ糖タンパクレセプターのリガンドとなるガラクトースの混合溶液をリガンド溶液とし、共固定した F/G デンドリマーを用いたところ、スフェロイドの接着が維持された。この、F/G デンドリマー上で培養したラット初代



八木清仁

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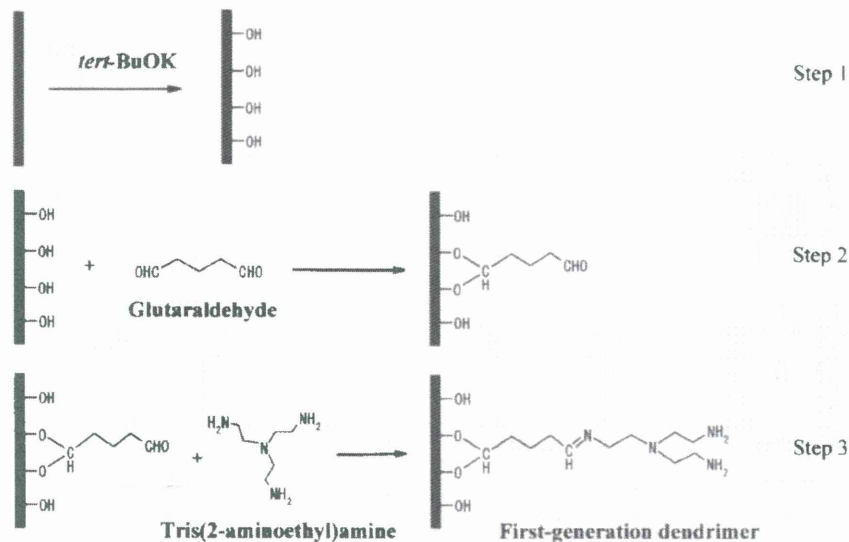


Fig. 2. Schematic Illustration of Dendrimer Immobilization onto the Surface of Polystyrene Plate

肝細胞は、リガンドがフルクトース、ガラクトース単独のものに比べ、肝特異的機能であるウレア合成能が向上し、アルブミン遺伝子の発現も維持しており、機能維持についても優れていることが確認できた。また、共焦点レーザー顕微鏡による解析から、リガンドのないデンドリマー上のスフェロイド内部の細胞はアポトーシスを起こしているのに対し、F/Gデンドリマー上のスフェロイドではアポトーシスが抑制されていることを確認した。⁷⁾

リガンド修飾デンドリマーを基材表面上に固定し、細胞培養に用いるアイデアは筆者ら独自のものである。これまでの検討から、細胞毎に異なるリガンドを用いることで、リガンド修飾デンドリマーによって最適な細胞培養表面の創出が可能であることを示してきた。大阪大学基礎工学研究科の田谷正仁教授のグループは筆者らとの共同研究においてデンドリマーの密度を変化させることにより軟骨細胞の形態、分化機能を制御することに成功した。^{8,9)} またD-グルコースと **Epidermal growth factor** をリガンドとして用いると細胞の増殖及び運動性を亢進できることを報告している。¹⁰⁾ さらに大阪大学医学系研究科の宮崎純一教授のグループはD-グルコースをデンドリマーにより培養表面に提示するとES細胞の未分化能が有意に維持されることを見出ししている。¹¹⁾ このようにリガンド修飾デンドリマーは、細胞毎に最適化したカスタムメイドの培養基材表面創出のツールとなり、組織工学全般の発展に大きく貢

献することが期待される。

3. 3次元培養による肝機能制御

ラジアルフロー型バイオリクター (RFB) は一般に円筒形のリアクター内に細胞接着用の担体を充填し円筒周囲より培養液あるいは血漿が中心部に向けて流れる構造をしている。RFBは従来のリアクターに比べ、灌流液の流速による剪断力が弱いため細胞障害が少なく、酸素や栄養物の供給がより均一に行われることが知られている。これまで動物細胞が 1×10^8 cells/ml 以上の高密度で培養可能であることが示されている。¹²⁾ 筆者らはRFBをバイオ人工肝臓に応用することを目的とし多孔質ガラスビーズ (シランビーズ) を担体として肝細胞の灌流培養を行った。Figure 3にRFBを用いた培養システム図を示した。筆者らは骨髄間質細胞を肝細胞の生存性を延長し機能を強化するための支援細胞として選択した。骨髄間質細胞は骨髄においてコラーゲン、フィブロネクチンを始めとする細胞外マトリクスや種々の増殖因子を産生し、造血幹細胞や血球系の細胞の維持に重要な役割を演じていることが知られている。プラスチックディッシュによる2次元培養の実験より骨髄由来の間質細胞が肝細胞の機能を維持する効果があることを既に明らかにしており、¹³⁾ その効果が3次元担体を用いたRFBにおいて発現するか否かを検討した。

実験はSD系雄性ラット由来の細胞を用いて行った。肝細胞は生体内においては肝再生時旺盛に増殖

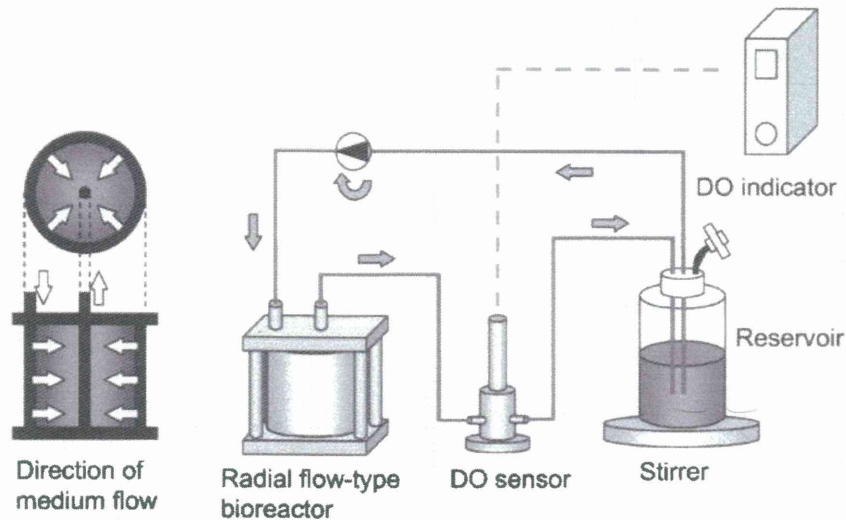


Fig. 3. Perfusion Culture of Hepatocytes by Radial Flow-type Bioreactor

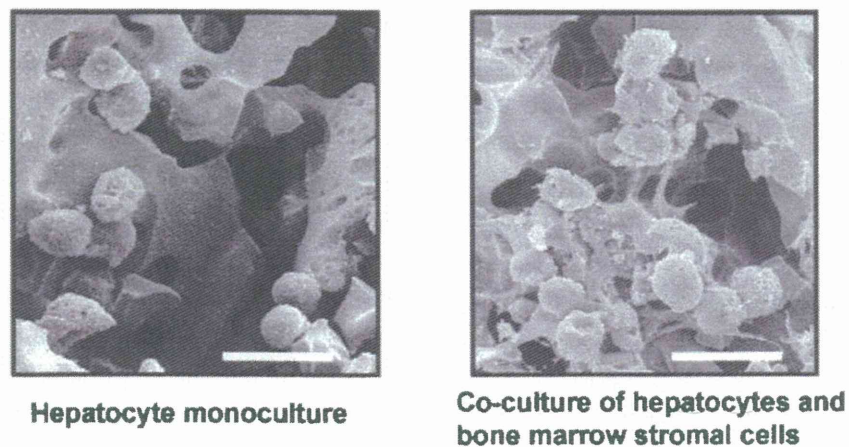


Fig. 4. Electron Micrographs of Hepatocytes Cultured in Silan Beads
White bar indicates 50 μm .

するが *in vitro* では増殖させることは困難であるため初期接着した細胞数を維持することが重要となる。一方骨髄間質細胞は *in vitro* で活発に増殖するため、共培養時には最初に骨髄間質細胞を播種し担体上でサブコンフルエントに達するまで培養した後肝細胞を播種した。Figure 4 にシランビーズを用いた肝細胞単独培養、骨髄間質細胞との共培養を行った際の電子顕微鏡写真を示した。単独培養では直径約 15-20 ミクロンの球状をした肝細胞がシランビーズ上に接着している様子が観察される。一方共培養においてはシランビーズ上に伸展した骨髄間質細胞に肝細胞が接着していた。そしてそれぞれのシランビーズを充填した RFB を用いて 4 日間の連続

灌流培養を行った。その間 24 時間おきにサンプリングを行い、肝特異機能である尿素合成能を評価した。その結果、対照として行った肝細胞単独の 2 次元培養では 4 日間で機能は約 9% に低下したが、RFB の 3 次元培養においては 29% までの低下にとどまった。したがって RFB による灌流培養の効果が示された。さらに骨髄間質細胞との共培養により 47% の機能が有意に維持され、肝細胞に対する効果が 2 次元培養のみならず RFB を用いた 3 次元培養においても発現することが明らかとなった。骨髄間質細胞は培養によって増幅することが可能でありヒト由来、あるいは患者自身の細胞を将来使用することも視野におくとバイオ人工肝臓の機能を強化し得

る細胞源として有望と考えている。さらには骨髄細胞中には肝細胞へ分化可能な間葉系幹細胞 (MSC) が存在することから患者由来の細胞を利用した薬物代謝評価系の構築も可能であり個の医療への応用も期待される。

4. 遺伝子導入による肝機能制御

これまで肝がん細胞にアンモニア代謝、薬物代謝に係わる個々の遺伝子を導入する試みは国内の他グループにより報告されている。^{14,15)} われわれは人工肝臓が担うべき数百という肝機能を考慮し細胞全体をグローバルに活性化し、かつ細胞死に対する抵抗性を付与することを目的として遺伝子導入を試みている。

チオレドキシンと呼ばれるタンパク質はリボヌクレオチドリダクターゼの生理的還元剤として発見されたが酸化ストレスやアポトーシスに対して抵抗性を付与するという機能が報告され注目を浴びている。^{16,17)} われわれはこのチオレドキシン遺伝子を肝細胞へ導入することにより生体外において引き起こされるストレス及びアポトーシスに対し抵抗性を獲得させることを試みた。肝細胞は生体外では増殖が困難であること、そして遺伝子導入効率を考慮しアデノウィルスをベクターとして用いることとした。ヒトチオレドキシン遺伝子を挿入した組換えアデノウィルスを作成しラット肝細胞へ感染させた。ヒトチオレドキシンが発現していることをウェスタンブロットで確認後、過酸化水素処理に対する抵抗性を調べた。1 mM 過酸化水素で 24 時間処理した後、アポトーシスを起こした細胞数を fluorescence activated cell sorting (FACS) により測定した。コントロールの肝細胞は約 80% がアポトーシスを起こしたのに対し、チオレドキシン遺伝子を導入した肝細胞は約 25% とアポトーシスに対して抵抗性を獲得したことが示された。また、通常のポリスチレンプレートで培養したときの寿命が延長されるか否かを調べたところ明らかな効果が観察された。尿素合成能も同時に維持されチオレドキシン遺伝子導入の有効性が示された。¹⁸⁾ リアクターへ充填する細胞へ当該遺伝子をウィルスベクターを用いて導入することも可能であり、またチオレドキシントランスジェニック動物を作成しその肝細胞をバイオ人工肝臓や医薬品開発のスクリーニング系に適用することも将来可能となるであろう。

5. 肝細胞源の検討

肝細胞源としてはヒトの細胞を用いることが理想的である。再生医療用の細胞源としてこれまで ES 細胞、骨髄細胞などが主に検討されてきたがわれわれは通常廃棄される組織から肝細胞へ分化可能な幹細胞を単離することができれば有用であると考えた。歯科領域では歯髄から MSC が単離されたことが報告されており、^{19,20)} 抜歯され廃棄される歯に着目した。虫歯の場合、病原菌が含まれ再生医療に適用することは困難であるため、歯科矯正時に抜歯される第 3 大臼歯、通称“親知らず”を用いることとした。矯正時に抜歯されるものは埋伏した状態であり、未分化な歯胚組織が維持されている可能性が高く、分化が進むと象牙質、歯髄となる歯乳頭組織には有用な MSC が存在することが予想された。そこでインフォームドコンセントを得た後、破棄された親知らずより歯乳頭組織を採取し MSC のクローン単離を試みた。

歯乳頭組織をはさみで細かく切断し、コラゲナーゼにより細胞を分散後組織培養用ディッシュに播種し α -MEM を用いて培養を行った。接着性の細胞を回収し FACS を用いて 96 穴プレートの 1 ウェルあたり 1 つの細胞が入るように播種した。単一細胞からコロニー形成したものを継代しさらに増殖させ、 2×10^4 cells を分化能の評価に使用し、残りの細胞を凍結保存した。カルセインを利用した骨分化能を指標として幹細胞としての特性を有するクローンの選択を行った結果、コロニー形成能を有するものの約 30% が骨分化能を発現した。その中から特に高い骨分化能を示したクローンを用いて以下の検討を行った。

肝細胞への分化誘導には Hamazaki らの方法²¹⁾ に準じ、HGF、デキサメタゾン、ITS に加えて線維芽細胞増殖因子 (FGF)、オンコスタチン M (OSM) を用いた。培養初期には細長い線維芽細胞用の形態であるが分化誘導を継続するにつれ、2 週間後にはサイズの大きい多角の形態へと変化した。RT-PCR による解析の結果、分化誘導 10 日でアルブミン遺伝子の発現が観察され、逆に初期分化マーカーである AFP 遺伝子発現は減少する傾向にあった。次に肝障害ラットを用いて移植の効果を検討した。

ヒト細胞を移植するため拒絶反応を起こさない免疫不全のヌードラットを使用した。9 週齢のフィッ

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

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

6. おわりに

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

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

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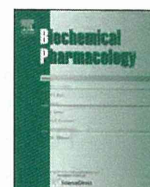
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A claudin-4 modulator enhances the mucosal absorption of a biologically active peptide

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ABSTRACT

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

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1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Materials

Human parathyroid hormone derivative (hPTH(1-34)) was prepared as described previously [23]. n-Dodecyl- β -D-maltoside (DDM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Anti-claudin antibodies and anti-his-tag antibody were obtained from Invitrogen (Carlsbad, CA) and EMD Chemicals Inc. (Darmstadt, Germany), respectively. CM5 sensor chips, amine-coupling reagents (N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), and ethanolamine-HCl) and HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P20) were obtained from GE Healthcare (Buckinghamshire, UK). All reagents used were of research grade.

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

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

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

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

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

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

The claudin-displaying BV were diluted with TBS and adsorbed to the wells of 96-well immunoplates (Nunc, Roskilde, Denmark) overnight at 4°C . The wells were washed with PBS and blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) for 2 h at room temperature. C-CPE or C-CPE derivatives were added to the wells and incubated for an additional 2 h at room temperature. The wells were washed with PBS and incubated with anti-his-tag antibody for 2 h at room temperature. The immuno-reactive proteins were detected by a horseradish peroxidase-labeled secondary antibody with 3,3',5,5'-tetramethylbenzidine as a substrate. The reaction was terminated by the addition of 0.5 M H_2SO_4 , and the immuno-reactive proteins were measured at 450 nm.

2.5. Preparation of recombinant claudin-4 protein

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

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

2.6. Surface plasmon resonance (SPR) analysis

SPR measurements were performed with a Biacore T100 instrument (GE Healthcare). Amine-coupling chemistry was used to immobilize claudin-4 at 25 °C on a CM5 sensor chip surface docked in a Biacore T100 and equilibrated with HBS-EP+. The carboxymethyl surface of the CM5 chip was activated for 2 min with a 1:1 ratio of 0.4 M EDC and 0.1 M NHS at a flow rate of 10 μ l/min. Claudin-4 was diluted to 2.5 μ g/ml in 10 mM MES buffer (pH 6.5) and injected for 2 min over the surface at a flow rate of 10 μ l/min. Excess activated groups were blocked by a 5-min injection of 1 M ethanolamine (pH 8.5) at a flow rate of 10 μ l/min. Approximately 1000 RU of claudin-4 was immobilized by using this protocol. Single-cycle kinetics experiments were performed at 25 °C with a flow rate of 30 μ l/min [27]. C-CPE or its derivatives were serially diluted (1.25, 2.5, 5, 10 and 20 nM) in running buffer (HBS-EP+). Within a single binding cycle, samples of C-CPE or its derivatives were injected sequentially in order of increasing concentration over both the ligand and the reference surfaces. The reference surface, an unmodified flowcell, was used to correct for systematic noise and instrumental drift. Also, prior to the binding cycle for C-CPE or its derivatives, buffer was injected. These “blank” responses were used as a double-reference for the binding data [28]. The sensorgrams were globally fitted by using a 1:1 binding model to determine k_a , k_d and K_D values with the Biacore T100 Evaluation Software version 2.0.1.

2.7. Transepithelial electric resistance (TEER) assay

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

2.8. In situ loop assay

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

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

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

2.9. Nasal and pulmonary absorption assay

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

2.10. Statistical analysis

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

3. Results

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

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

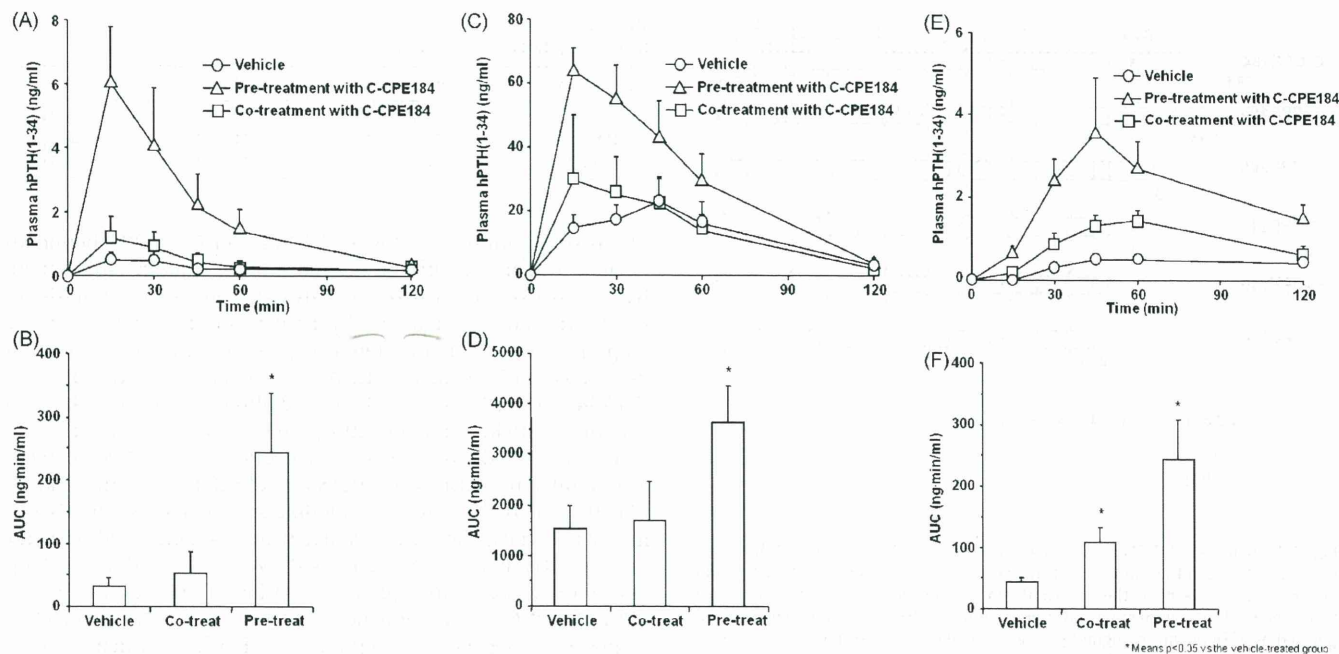


Fig. 1. Effect of C-CPE184 on mucosal absorption of hPTH(1–34) in rats. (A, B) Jejunal absorption of hPTH(1–34). Jejunum was co-treated with hPTH(1–34) (100 μ g) and C-CPE184 (20 μ g), or jejunum was treated with hPTH(1–34) 4 h after treatment with C-CPE184. (C, D) Pulmonary absorption of hPTH(1–34).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

Table 1

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

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

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

Data are means \pm SE.

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

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

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

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

Table 2

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

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

^a Solvent is PBS.

^b Insol., insoluble.

[26]. Although C-CPE194 is a claudin-4 binder, whether C-CPE194 modulates the TJ-barrier remains unclear. C-CPE194 contains nine β -sheets and one α -helix, and its 16 C-terminal amino acids are believed to comprise the claudin-4-binding region (Fig. 2A) [26,29]. Based on this information, we prepared five different N-terminal-truncated C-CPE184 derivatives: C-CPE194, which lacks the 10 N-terminal amino acids; C-CPE205, which is truncated prior to the β 1-sheet; C-CPE212, which is truncated after the β 1-sheet; C-CPE219, which is truncated after the α -helix; and C-CPE224, which is truncated before the β 2-sheet (Fig. 2A). The C-CPEs were expressed in *E. coli* (Fig. 2B). The solubility of C-CPE194 (>10 mg/ml) and C-CPE205 (>4 mg/ml) in PBS was greater than that of C-CPE184 (<0.3 mg/ml) (Table 2). However, C-CPE212, C-CPE219 and C-CPE224 formed solid inclusion bodies in *E. coli*, and these inclusion bodies could not be dissolved without 2 M urea. Therefore, further experiments were performed with C-CPE184, C-CPE194 and C-CPE205.

3.3. Characterization of C-CPE194 and C-CPE205

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

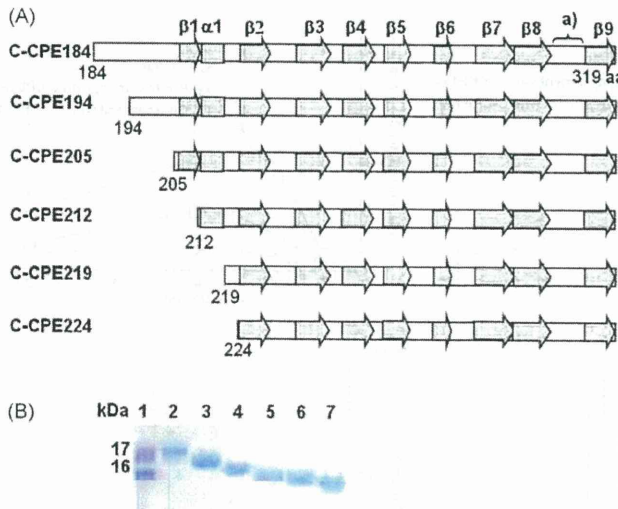


Fig. 2. Preparation of C-CPEs. (A) Schematic structure of C-CPEs. Van Itallie et al. determined the 3-dimensional structure of C-CPE194 containing nine β -sheets and one α -helix [26]. Based on the structural information, we designed five N-terminal truncated C-CPE184 derivatives. (B) CBB staining. C-CPEs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

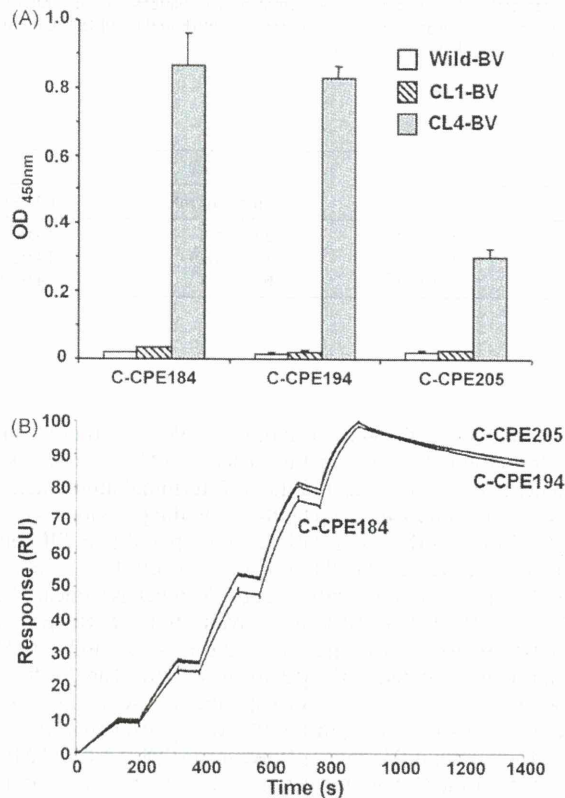


Fig. 3. Interaction of C-CPEs with claudin. (A) ELISA. The immunoplate was coated with wild-type BV (Wild-BV), claudin-1-displaying BV (CL1-BV) or claudin-4-displaying BV (CL4-BV), and then C-CPEs were added to the well. C-CPEs bound to BVs were detected by the addition of anti-his tag antibody and a labeled secondary antibody. Data are means \pm SD ($n = 3$). (B) SPR assay. Claudin-4 was immobilized on a CM5 sensor chip by the amine-coupling method. C-CPEs were injected sequentially at concentrations of 1.25, 2.5, 5, 10 and 20 nM. The association phase was monitored for 120 s at a flow rate of 10 μ l/min, and the dissociation phase was followed for 600 s at the same flow rate. The maximum values of response (Rmax) for all curves were compensated to 100 RU.

Table 3

Binding kinetics of C-CPEs to claudin-4.

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

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

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

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

4. Discussion

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

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

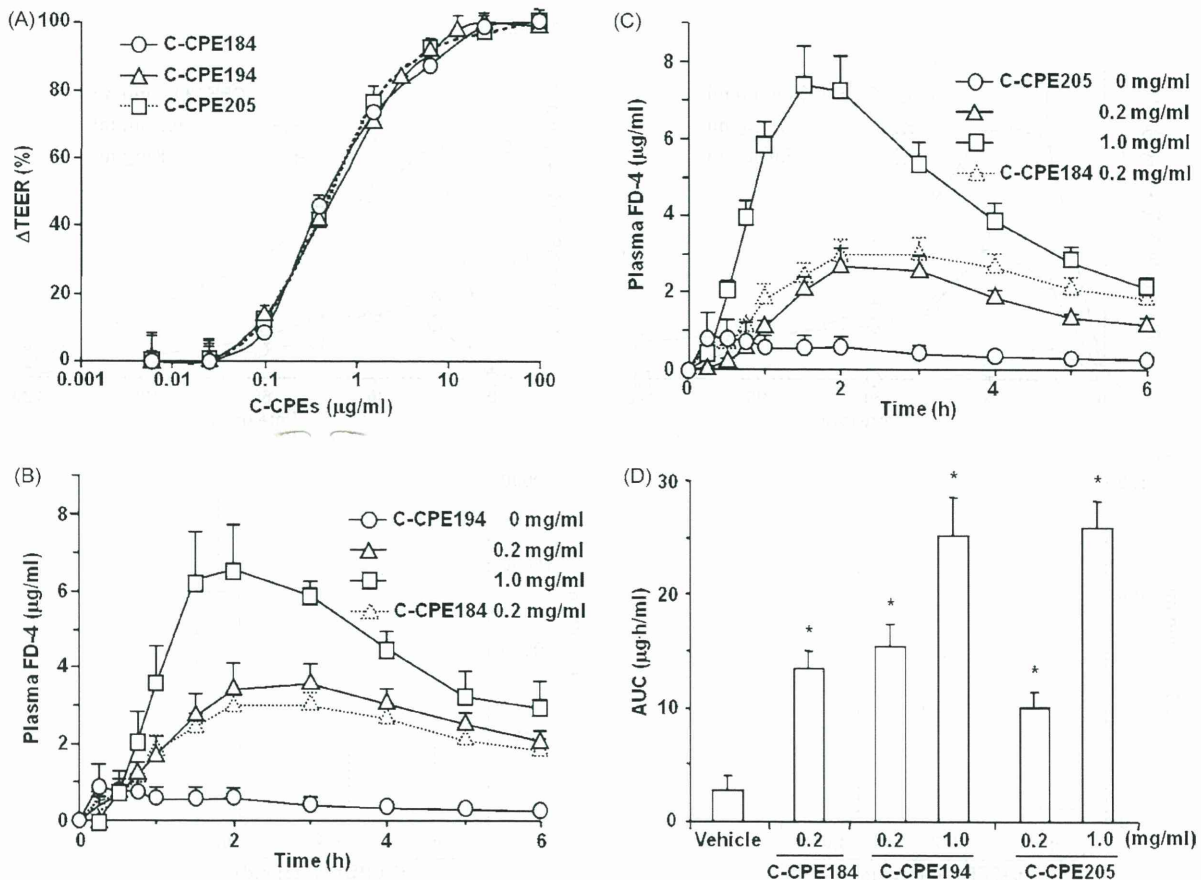


Fig. 4. Modulation of the TJ-barrier by C-CPes. (A) Caco-2 cells were seeded on a BioCoat™. When TJ-barriers were developed and the cell sheets reached a plateau in their TEER value, C-CPes were added to the wells from the basal side at the indicated concentration. After 18 h, the TEER was measured.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

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

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

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

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

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

Derivatives	EC50 values ^a
C-CPE184	0.49 μ g/ml
C-CPE194	0.57 μ g/ml
C-CPE205	0.51 μ g/ml

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

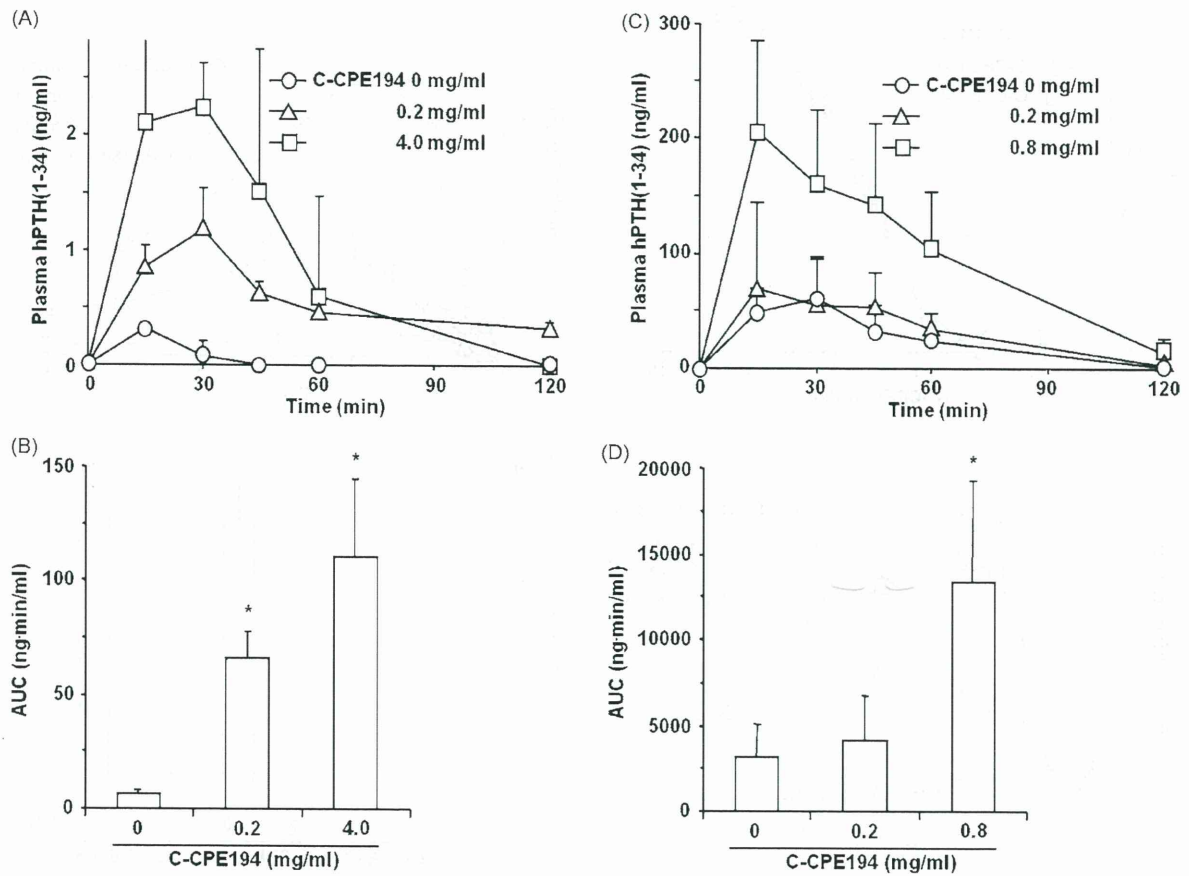


Fig. 5. Mucosal absorption of hPTH(1-34) by a claudin-4 modulator. (A, B) Jejunal absorption of hPTH(1-34). Rat jejunum was treated with hPTH(1-34) (100 µg) and C-CPE194 at the indicated doses. Time-course changes in plasma hPTH(1-34) (A) and AUC from 0 to 120 min (B) were analyzed. (C, D) Pulmonary absorption of hPTH(1-34). hPTH(1-34) (150 µg) and C-CPE194 at the indicated doses were pulmonary administered, and time-course changes in plasma hPTH(1-34) concentration (C) and AUC from 0 to 120 min (D) were analyzed. Data are mean ± SE (n = 3). *Significantly different from the vehicle-treated group (p < 0.05).

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

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

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

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

C-CPE194 (mg/ml)	Jejunum		C-CPE194 (mg/ml)	Pulmonary	
	Cmax (ng/ml)	BA (%) ^a		Cmax (ng/ml)	BA (%)
0	0.3 ± 0.0	0.1 ± 0.0	0	62.3 ± 32.8	26.5 ± 17.4
0.2	1.2 ± 0.4*	0.8 ± 0.2**	0.2	78.7 ± 66.1	34.1 ± 21.6
4.0	2.8 ± 0.2*	1.3 ± 0.3**	0.8	205.2 ± 79.4*	100.6 ± 39.3*

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

Data are mean ± SE.

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

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

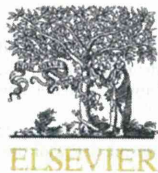
In summary, we found that claudin-4 modulator enhanced the jejunal, pulmonary and nasal absorption of a peptide drug. This report is the first to indicate that a claudin-4 modulator may be a mucosal-absorption enhancer of biologics.

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

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ABSTRACT

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

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1. Introduction

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

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

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

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

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