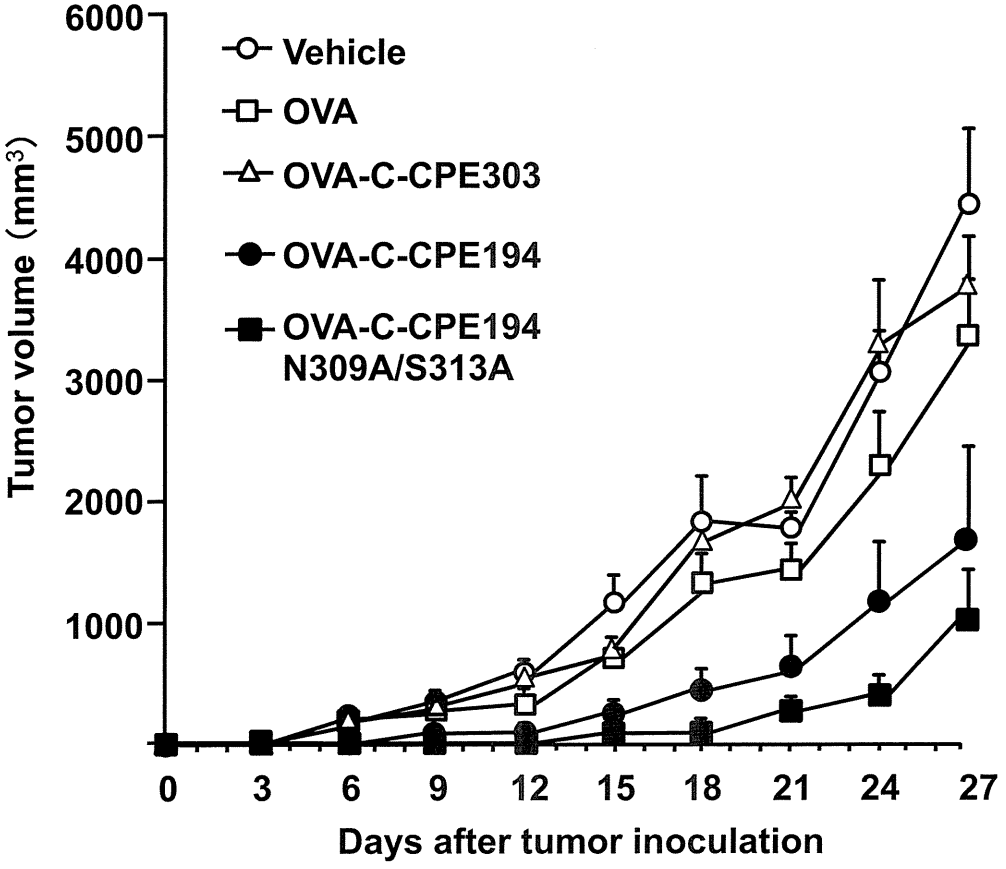
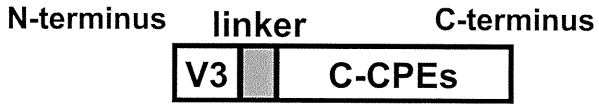


Fig.7 Anti-tumor activity induced by immunization with OVA-C-CPE 194 N309A/S313A in an E.G7-OVA cancer model.
C57BL/6 mice were nasally immunized with vehicle, OVA, OVA-C-CPE 303, OVA-C-CPE 194 or OVA-C-CPE 194 N309A/S313A (5 mg of OVA) once a week for three weeks. Seven days after the last immunization, the mice were injected s.c. on the right back with 1×10^6 E.G7-OVA cells. The tumor growth was monitored by measuring two diameters, and the tumor volumes was calculated as $a \times b \times b/2$, where a is the maximum diameter of the tumor and b is the minimum diameter of the tumor. Data are means ± SEM (n=5~6). The results are representative of three independent experiments.

A



B

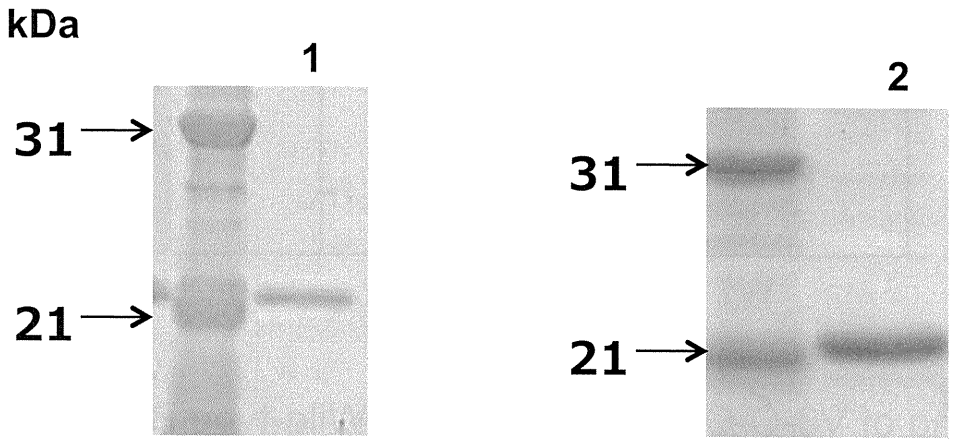


Fig.8 Preparation of V3-C-CPE mutant.
A) Schematic illustration of V3-C-CPE mutant. V3 was fused with C-CPE mutant at the N-terminal of C-CPE mutant, resulting in V3-C-CPE mutant. B) Purification of V3-C-CPE mutant. V3-C-CPE mutant was expressed in *E. coli* and isolated by anti-His tag affinity chromatography. The purification of V3-C-CPE mutant was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB) and by immunoblotting with an anti-his-tag antibody. Lane1, V3-C-CPE 303; lane2, V3-C-CPE 194N309A/S313A.

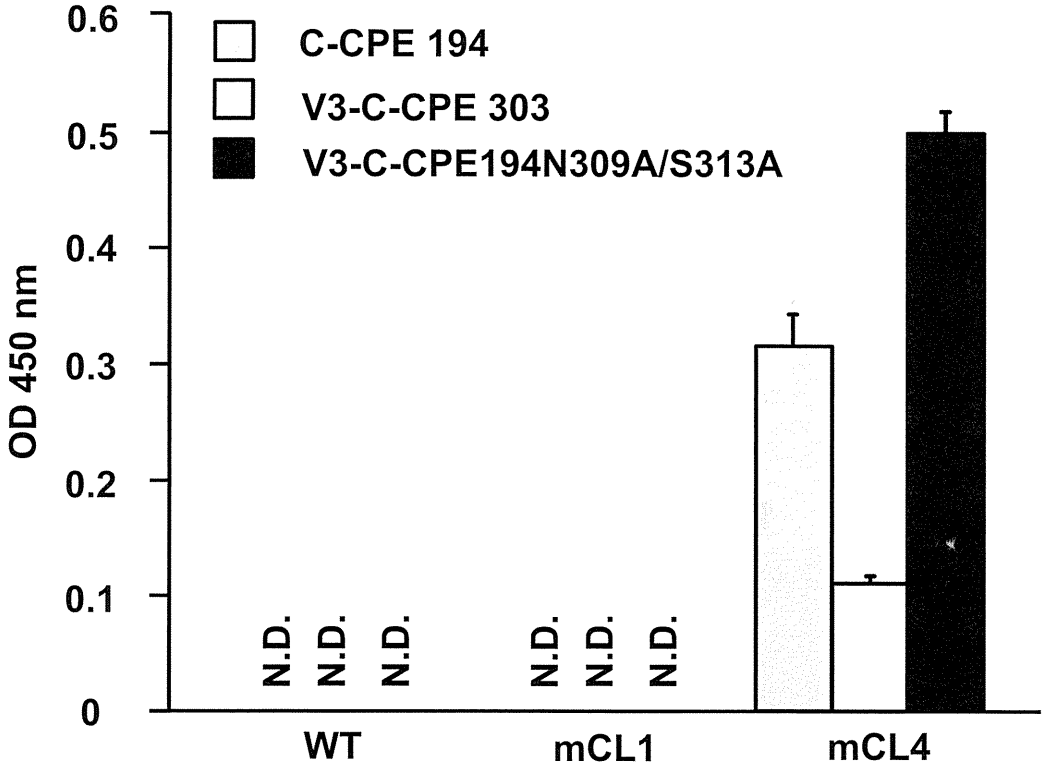


Fig.9 Binding of V3-C-CPEs to claudin-4. Wild-type BV (WT-BV), mouse claudin-1 displaying BV (mCL1-BV) or -4 (mCL4-BV) was absorbed onto immunoplate, and then V3-C-CPEs were added to the well. V3-C-CPEs bound to BV was detected by an anti-his-tag antibody followed by goat anti-mouse IgG (H+L)-HRP. Data are means ± SD (n=4)

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分担研究報告書

「パイエル板指向性分子の作製・機能解析」

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研究要旨

本研究は依然として猛威を振るっている感染症に対する有効な予防法である経口ワクチンの開発を目的としている。経口ワクチンは非侵襲性投与が可能であり、全身免疫および粘膜免疫の双方を誘導可能であることから理想的なワクチン形態であるが、腸管粘膜免疫組織への効率の良い抗原送達法の確立、消化酵素による抗原分解回避などの障壁があり、開発が立ち遅れている。近年、腸管粘膜免疫組織パイエル板を覆う上皮細胞層に claudin(CL)-4 が高発現していること、さらにパイエル板において抗原取り込みを担う M 細胞に CL-4 が高発現していることが示され、CL-4 を標的とした経口ワクチン開発の可能性が示唆された。そこで本研究では、CL-4 へ結合性を示す、ウエルシュ菌下痢毒素の受容体結合領域断片である C-CPE と消化酵素耐性能をもつリポソーム技術を融合することにより、経口ワクチン開発に資するパイエル板へのワクチン抗原送達方法の開発を試みる。

これまでの検討により、既存の C-CPE では腸管粘膜免疫組織への抗原送達率が低いことが想定されたことから、CL-4 高親和性の C-CPE194N309A/S313A が創出された。本年度は、C-CPE 194N309A/S313A を用い、本 C-CPE 変異体とモデル抗原である OVA を連結した OVA 融合 C-CPE 194N309A/S313A を作製し、その CL-4 結合性を検証した。

A. 研究目的

新型インフルエンザの流行でも分かるように、感染症は僅か数日間で世界中に蔓延してしまう状況にあり、少子高齢社会の我が国では安全保障の観点から感染症予防法の確立が喫緊の課題となっている。病原性微生物の侵入門戸である粘膜面に感染防御網を構築する粘膜ワクチンは唯一理想的な感染予防技術であり、本研究で開発を試みる経口ワクチンは国民の健康確保に貢献すると考えている。

最近、感染症（年間死者数 2000 万人）や癌（年間死者数 700 万人）のみならず、アルツハイマー病（患者数 2000 万人）やリュウマチ（患者数 600 万人）に対するワクチン療法の有用性が示され、世界のメガファーマが次々とワクチン開発に乗り出してきている。前述したように、患者の生活の質（QOL）およびワクチン活性を考慮すると経口ワクチンが理想的なワクチンであるものの、腸管粘膜免疫組織パイエル板（PP）への抗原送達技術開発の遅延から、依然としてポリオ生ワクチンしか実用化されていない。本研究は、独自の PP 指向性分子を創出し、初めての PP 指向性経口ワクチンの創出を試みるものであり、我が国のワクチン産業に特徴あるワクチン開発力を寄与できる。さらに、ワクチンは免疫記憶によりわずか数回の投与で予防・治療効果を期待できることから、増加の一途を辿るわが国の医療費を削減するなど、本研究成果は多方面から厚生労働行政に資するものである。

本年度は、既存の C-CPE に比して結合性が 10 倍向上した C-CPE 194N309A/S313A を用い、本 C-CPE 変異体とモデル抗原である OVA を連結

した融合蛋白質を作製し、その CL-4 結合性を検証した。

B. 研究方法

B-1. OVA 融合 C-CPE194 N309A/S313A の作製

OVA 融合 C-CPE194 N309A/S313A 発現 plasmid の作製

C-CPE 194 N309A/S313A の遺伝子を pET16b に組み込んだ pET-C-CPE 194 N309A/S313A プラスミドを用いた。pET-C-CPE 194 N309A/S313A を *Nde* I (New England Biolabs., Inc) にて 37℃、120 分処理し、フェノール/クロロホルム抽出、エタノール沈殿を行った。一方、2 つの相補的なオリゴ (Fuka-25:5'-tataggtacccgggactagttaattaagggaggaggaggatctggaggaggaggatctggaggagc-3', Fuka-26:5'-tagctcctccagatcctcctcctccagatcctcctcctccctt aattaactagtcccgggtaccta-3') をハイブリダイズさせて作製した、マルチクローニングサイトと両端に *Nde* I binding サイトを持つ DNA を作製した。双方を T4 DNA ligase (New England Biolabs., Inc) を用いて 16℃にて一晩ライゲーション反応を行った。得られたライゲーション産物をフェノール/クロロホルム抽出、エタノール沈殿した後 *Nde* I を用いて 37 ℃、2 時間処理した。ライゲーション産物と大腸菌 DH5α (TOYOBO, Co., Ltd, Japan) を氷上で 15 分なじませた後、42℃で 40 秒 heat shock を行い、氷上で 3 分間静置した。その後 SOC 培地を添加し 37℃にて 50 分培養した後、100 μg/ml ampicilin sodium (SIGMA aldrich Japan Co., Ltd) を添加した LB 培地 (LA 培地) プレートに播種し一晩培養した。LA 培地を 3 ml 分注した Sterile Culture Tubes (IWAKIGLASS, Co., Ltd) に 1 コロニーずつピックアップし、一晩振盪培養した後、遠心分離し大腸菌を回収した。QIA prep Spin Miniprep kit (50) (QIAGEN Sience, USA) にて大腸菌より plasmid を精製した。得られた plasmid をシークエンス解析し、目的の遺伝子配列と一致することを確認した。

上記の操作で得たプラスミドに対し *Kpn* I, *Pac* I (New England Biolabs., Inc) にて 37℃、120 分制限酵素処理し、フェノール/クロロホルム抽出、エタノール沈殿を行った。一方、OVA-C-CPE plasmid を精製し、*Kpn* I, *Pac* I を用いて 37℃、120 分処理し、フェノール/クロロホルム抽出、エタノール沈殿を行った。1% TAE ゲルにて電気泳動を行い、目的の OVA のバンドを切り出し、精製を行った。双方の DNA を T4 DNA ligase を用いて 16 ℃にて一晩ライゲーション反応を行った。得られたライゲーション産物をフェノール/クロロホルム抽出、エタノール沈殿した後 *Spe* I (New England Biolabs., Inc) で 37℃、120 分処理した。ライゲーション産物を大腸菌 DH5α にトランスフォーメーションし (方法は前段落と同様) 、大腸菌を回収した。QIA prep Spin Miniprep kit (50) にて大腸菌より plasmid を精製した。得られたサンプルについてシークエンス解析し、His-OVA-C-CPE 194 N309A/S313A をコードしたプラスミドを得た。

B-2. OVA 融合 C-CPE194 N309A/S313A タンパク質の作製確認

OVA-C-CPE 194 N309A/S313A 発現 plasmid 1 μl を BL21(DE3)(Novagen, Co., Ltd) 10 μl に加え、氷上で 15 分なじませ、40 秒間 heat shock を行い 3 分間氷上で静置した後、SOC 培地 100 μl を加え 37℃で 1 時間培養し、LA プレートに播種し一晩培養した。LA 培地 3 ml を分注した Sterile Culture Tubes に 1 コロニーをピックアップし、37℃で一晩振盪培養し翌日 LA 培地を 2 ml ずつ分注した Sterile Culture Tubes を 4 本用意し、大腸菌培養液を 200 μl ずつ加え、37 ℃で 3 時間振とう培養した。その後、isopropyl-β-D (-) thiogalactopyranoside (IPTG, WaKo Pure Chemicals Ind., Japan) を終濃度 0、0.25、0.5、1.0 mM となるように添加し、30 または 37℃で 3 時間振とう培養した。遠心分離により大腸菌を回収後、200 μl の 1×SDS (62.5 mM Tris-HCl, 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.001% bromophenol blue) に懸濁し、氷冷しながら超音波処理 20 秒×3 回行

い大腸菌を破砕した。4 ℃, 14000 rpm で 10 分間遠心分離し、上清を回収して 99℃で 5 分間加熱しサンプルとした。10% polyacrylamide gel を用いて 30 mA で電気泳動 (SDS-PAGE) を行い、CBB (Bio-Rad laboratories, Inc., USA) で 1 時間染色し MilliQ で脱色した後、62 kDa 付近の OVA-C-CPE 194 N309A/S313A が多く産生されている IPTG 濃度を最適なものとした。

OVA-C-CPE 194 N309A/S313A 発現 plasmid 1 μl を BL21(DE3)(Novagen, Co., Ltd) 10 μl に加え、氷上で 15 分なじませ、40 秒間 heat shock を行い 3 分間氷上で静置した後、SOC 培地 100 μl を加え 37℃で 1 時間培養し、LA プレートに播種し一晩培養した。大腸菌 10 コロニー程度を LA 培地 50 ml に移し、37℃で一晩培養した (少量培養)。翌日 TA (TERRIFIC BROTH/amp , Invitrogen , Co., Ltd) 培地 500 ml に大腸菌培養液全てを移し、37℃で 3 時間振とう培養した。その後、決定した発現誘導条件に従い IPTG を添加し 37℃で 3 時間振とう培養した (大量培養) 後、10,000 rpm で 1 分間遠心分離し大腸菌を回収した。

500 ml の大腸菌培養液のうち 100 ml を可溶化条件の検討に用い、400 ml は精製条件の検討に用いた。100 ml culture の大腸菌を buffer A (10 mM Tris-HCl (pH 8.0), 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethane sulfonyl fluoride, 1 mM 2-mercaptoethanol, 10% glycerol) 1 ml に懸濁し、氷冷しながら超音波処理 40 秒を 3 回行い大腸菌を破砕した。4℃, 14,000 rpm で 15 分間遠心分離し上清を回収後、沈殿に 2 % TrironX-100 含有 buffer A を 1 ml 加え超音波処理を行った。遠心分離後沈殿に 8 M Urea 含有 buffer A を 1 ml 加え超音波処理をした。遠心分離後上清を回収し、沈殿に buffer A を 1 ml 加え超音波処理を行い懸濁させた。それぞれの溶液画分 20 μl に 4×SDS を 6.7 μl 加え、99℃で加熱しサンプルとした。10 % polyacrylamide gel を用いて SDS-PAGE を行い CBB 染色した後、OVA-C-CPE 194 N309A/S313A が可溶化した画分の buffer を可溶 buffer とした。

OVA-C-CPE 194 N309A/S313A 発現 plasmid 1 μl を BL21 10 μl に加え、氷上で 15 分間なじませ、45 秒間 heat shock を行い 15 分間氷上で静置したのち、SOC 培地 100 μl を加え 37℃で 45 分間培養し、LA プレートに播種し一晩培養した。大腸菌 10 コロニー程度を LA 培地 100 ml に移し、37℃で一晩培養した。翌日 TA 培地 1 リットルに大腸菌培養液すべてを移し、37℃で 2 時間振とう培養した後、最適濃度の IPTG を添加し 37℃で 3 時間振とう培養した。その後 10,000 rpm で 1 分間遠心分離して大腸菌を回収し、−20℃で凍結保存した。

大腸菌を氷上で溶解した後、可溶化条件の検討結果に従い、buffer A を用いて OVA-C-CPE 194 N309A/S313A の可溶化を行った。buffer A を 1 ml/100 ml culture の割合で添加し、40 秒間の超音波処理を 3 回行った後、14000 rpm , 15 分にて遠心分離を行い、上清を回収した。予め 6 M guanidine/EDTA , MilliQ , $NiSO_4$, buffer A を順に流して平衡化しておいた HiTrap™ Chelating HP (GE Healthcare) にサンプルを流し、OVA-C-CPE 194 N309A/S313A を吸着させた。100 μm の imidazol 溶液 10 ml で洗浄した後、400 μM の imidazol 溶液 10 ml で OVA-C-CPE 194 N309A/S313A を溶出させ、溶出液を 1 ml ずつ分取した。

OVA-C-CPE 194 N309A/S313A が溶解している buffer を PBS (-) (137 mM NaCl , 2.68 mM KCl , 8.14 mM Na_2HPO_4 , 1.15 mM KH_2PO_4) に置換するため PD-10 column (GE Healthcare Bio-Sciences Corp., USA) を用いた。あらかじめ PD-10 column に PBS を 30 ml 流して平衡化しておき、HiTrap™ Chelating HP で得た溶出液 1 ml を流した。PBS を 500 μl ずつ流して PD-10 column から溶出液を分取した。次にウシ血清アルブミン (PIERCE Biotechnology Inc., USA) を標準液として BCA™ Protein Assay Kit (PIERCE Biotechnology Inc., USA) を用い、560 nm における吸光度を測定し、OVA-C-CPE 194

N309A/S313A タンパク質の濃度を算出した。

B-3. OVA 融合 C-CPE194 N309A/S313A タンパク質の発現確認

上記の操作により得た、PBS に溶解した OVA-C-CPE 194 N309A/S313A 蛋白質を 100 μg/ml にて調整した。その溶液 20 μl に 4×SDS を 6.7 μl を加え、99 ℃で 5 分間加熱しサンプルとした。サンプルは 20 μl (蛋白量として 2 μg) アプライした。一方分子量マーカーとして Broad Range (BIO-RAD Laboratories, Inc., USA) を用いた。12% polyacrylamide gel を用いて 20 mA で電気泳動 (SDS-PAGE) を行い、CBB で 1 時間染色し MilliQ で脱色した後、62 kDa 付近に存在する OVA-C-CPE 194 N309A/S313A 蛋白質を確認した。

B-4. OVA 融合 C-CPE194 N309A/S313A の CL4 結合性

96 穴 ELISA plate (Greiner Bio-One GmbH, Germany) に BV-mClaudin-4 (ネガティブコントロールとして wild BV および BV-mClaudin-1) を 0.5 μg/well の条件で、4℃で一晩インキュベーションすることで固相化した。翌日、PBS(-)で 3 回洗浄後、1.6 % ブロックエース(DS PHARMA BIOMEDICAL, Japan) で室温、2 時間ブロッキングし、PBS(-) で 3 回洗浄した。続いて、C-CPE タンパク質量として 0.02 μg/well の条件で各種タンパク質を添加し、室温で 2 時間インキュベーションした。インキュベーション後、0.05% tween-PBS(-) (T-PBS) で 3 回洗浄後、1.6% ブロックエースで 3,000 倍に希釈した Mouse anti His-tag Ab (Zymed Laboratories Inc., Co, USA) を加え、室温で 2 時間反応させた。反応後、T-PBS で 3 回洗浄後、0.4% ブロックエースで 2,000 倍に希釈した Goat anti-Mouse IgG HRP conjugated を添加し、室温で 1 時間反応させた。反応後、T-PBS で 5 回洗浄後、TMB solution (Thermo Scientific, Rockford, IL) を加えた。20 分インキュベート後、2 M の硫酸を 100 μl/well 加え、吸光度を測定した。(450 nm , ref 595 nm)

C. 研究結果

結果は D 項にまとめて記載。

D. 考察

D-1. OVA 融合 C-CPE194 N309A/S313A タンパク質の作製

C-CPE194 N309A/S313A によるワクチン効果を検証するに当たり、モデル抗原として汎用されている卵白アルブミン OVA との融合タンパク質を作製した。まず、pET16b-C-CPE194 N309A/S313A plasimid に OVA 遺伝子を導入し、pET16b-OVA-C-CPE194 N309A/S313A plasimid を作製した。作製した plasmid を BL21 にトランスフォーメーションし、IPTG による蛋白質発現誘導および可溶バッファーの検討を行った。決定した IPTG 濃度および可溶バッファーを用い、AKTA によりタンパク質の精製を行った。作製したタンパク質を CBB 染色および Western blot 法により発現を確認した (Fig. 1)。予想される OVA-C-CPE194 N309A/S313A タンパク質の分子量付近にバンドが認められたことから、OVA-C-CPE194 N309A/S313A のタンパクが精製できていることを確認した。

D-2. OVA 融合 C-CPE194 N309A/S313A の CL 結合性

作製した OVA-C-CPE194 N309A/S313A の CL 結合性を CL 発現 BV を用いた ELISA 法および CL 発現細胞を用いた FACS 法により確認した。

WT、mCL1 および mCL4-BV をイムノチューブに固相化して、OVA-C-CPE194 N309A/S313A を添加し、HRP 標識抗体を用いて結合性を検証した。その結果、OVA-C-CPE194 N309A/S313A は OVA を付加した場合でも mCL4 に対して特異性を示し、結合性に変化は認められなかった。また、OVA-C-CPE194 N309A/S313A は OVA-C-CPE 194 に比して同等以上の mCL4 結合性を示した (Fig. 2)。さらに、CL 発現細胞を用いた FACS により

OVA-C-CPE194 N309A/S313A の結合性を検証した所、ELISA 法の結果と同様に mCL4 に対して特異性を示した(Fig. 3)。

E. 結論

本研究では CL4 を標的とした経口ワクチン開発の課題である粘膜免疫組織への抗原送達に関して C-CPE に比して CL4 結合性に優れる C-CPE 194N309A/S313A を用いて検証を行った。まず、モデル抗原として OVA と C-CPE 194N309A/S313A の融合タンパクを作製し、CL4 結合性を検証した結果、従来の OVA-C-CPE と比べて OVA- C-CPE 194N309A/S313A では CL4 結合性に優れたものであった。

F. 健康危険情報

該当事項なし

G. 研究発表

G-1 論文発表

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3. Shiraishi K, Endoh R, Furuhata H, Nishihara M, Suzuki R, Maruyama K, Oda Y, Jo J, Tabata Y, Yamamoto J, Yokoyama M.: A facile preparation method of a PFC-containing nano-sized emulsion for theranostics of solid tumors. Int J Pharm, 421: 379-387 (2011)
4. Un K, Kawakami S, Suzuki R, Maruyama K, Yamashita F, Hashida M. Suppression of Melanoma Growth and Metastasis by DNA Vaccination Using an Ultrasound-Responsive and Mannose-Modified Gene Carrier. Mol Pharm. 8: 543-554 (2011)
5. Negishi Y, Matsuo K, Endo-Takahashi Y, Suzuki K, Matsuki Y, Takagi N, Suzuki R, Maruyama K, Aramaki Y. Delivery of an Angiogenic Gene into Ischemic Muscle by Novel Bubble Liposomes Followed by Ultrasound Exposure. Pharm Res. 28: 712-719 (2011)
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H. 知的財産権の出願・登録状況

H-1 特許取得

なし

H-2 実用新案登録

なし

H-3 その他

なし

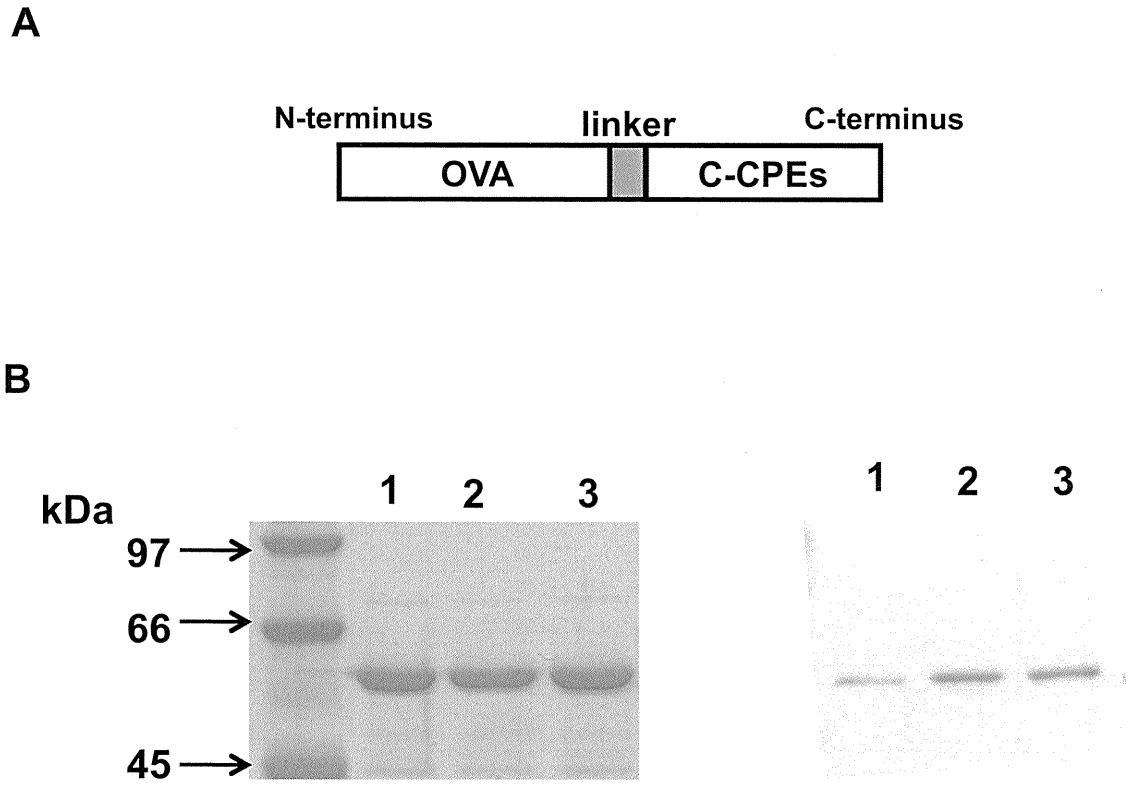

Fig.1 Preparation of OVA-C-CPE mutant.
A) Schematic illustration of OVA-C-CPE mutant. OVA was fused with C-CPE mutant at the N-terminal of C-CPE mutant, resulting in OVA-C-CPE mutant.
B) Purification of OVA-C-CPE mutant. OVA-C-CPE mutant was expressed in *E. coli* and isolated by anti-His tag affinity chromatography. The purification of OVA-C-CPE mutant was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue (CBB) and by immunoblotting with an anti-his-tag antibody. Lane1,OVA-C-CPE 303; lane2, OVA-C-CPE 194; lane3 OVA-C-CPE 194N309A/S313A.

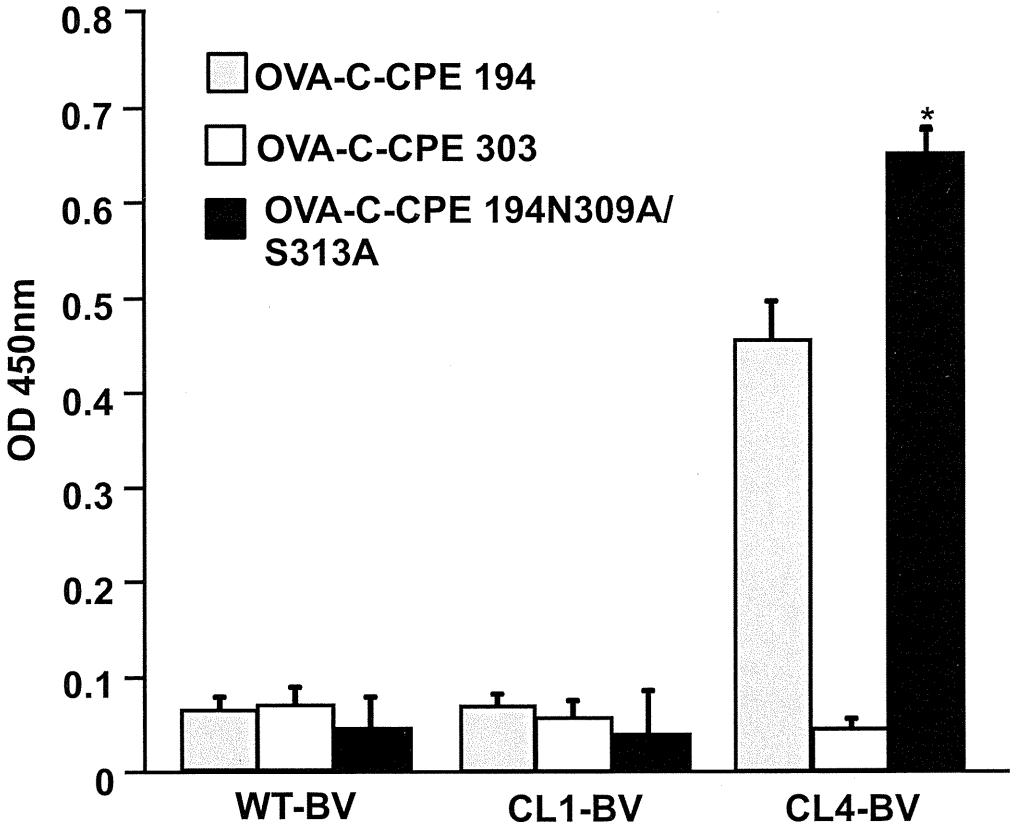

Fig.2. Binding of OVA-C-CPEs to claudin-4 displaying BV.
Wild-type BV (WT-BV), claudin-1 displaying BV (CL1-BV) or -4 (CL4-BV) was absorbed onto immunoplate, and then OVA-C-CPEs were added to the well. OVA-C-CPEs bound to BV was detected by an anti-his-tag antibody followed by goat anti-mouse IgG (H+L)-HRP.
Data are means ± SD (n=4).

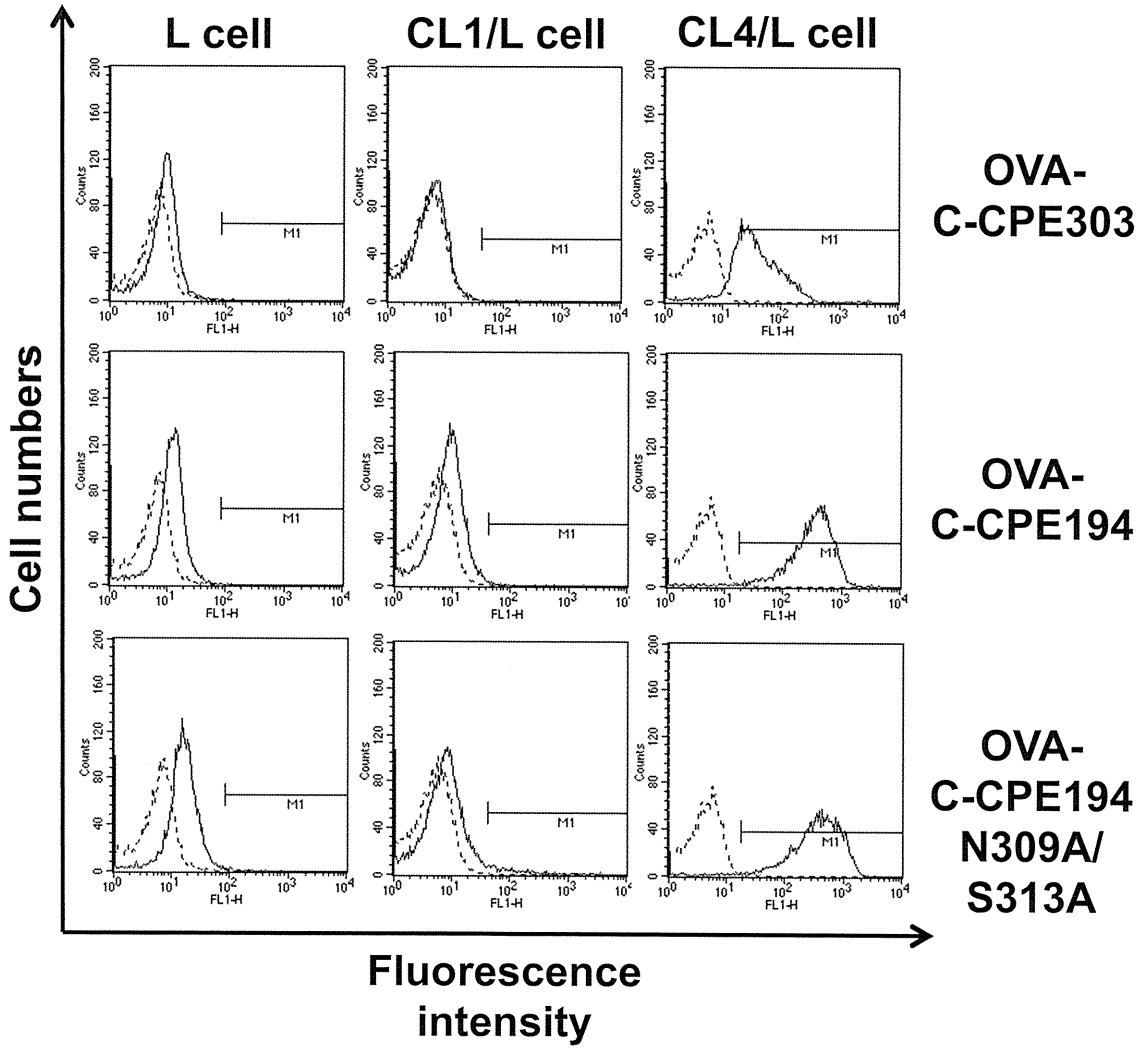

Fig. 3 Binding of OVA-C-CPEs to claudin-4-expressing cell. Claudin expressing L cells were incubated with OVA-C-CPEs followed by anti-his-tag antibody. Cells were stained with FITC-conjugated goat-anti mouse IgG (H+L) and analyzed by flow cytometry (solid line). Dotted line represents control cells incubated with the second and third step regent alone. (n=3)

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当事項なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Takahashi A, Kondoh M, Suzuki H, Watari A, Yagi K.	Pathological changes in tight junctions and potential applications into therapies.	*Drug Discov Today*			In press
Omata D, Negishi Y, Hagiwara S, Yamamura S, Endo-Takahashi Y, Suzuki R, Maruyama K, Nomizu M, Aramaki Y.	Bubble Liposomes and Ultrasound Promoted Endosomal Escape of TAT-PEG Liposomes as Gene Delivery Carriers.	Mol Pharm.	8:	2416-2423	2011
Un K, Kawakami S, Higuchi Y, Suzuki R, Maruyama K, Yamashita F, Hashida M.	Involvement of activated transcriptional process in efficient gene transfection using unmodified and mannose-modified bubble lipoplexes with ultrasound exposure.	J Control Release	156	355-363	2011
Shiraishi K, Endoh R, Furuhata H, Nishihara M, Suzuki R, Maruyama K, Oda Y, Jo J, Tabata Y, Yamamoto J, Yokoyama M	A facile preparation method of a PFC-containing nano-sized emulsion for theranostics of solid tumors.	Int J Pharm,	421	379-387	2011
Un K, Kawakami S, Suzuki R, Maruyama K, Yamashita F, Hashida M.	Suppression of Melanoma Growth and Metastasis by DNA Vaccination Using an Ultrasound-Responsive and Mannose-Modified Gene Carrier.	Mol Pharm.	8	543-554	2011

Negishi Y, Matsuo K, Endo-Takahashi Y, Suzuki K, Matsuki Y, Takagi N, Suzuki R, Maruyama K, Aramaki Y.	Delivery of an Angiogenic Gene into Ischemic Muscle by Novel Bubble Liposomes Followed by Ultrasound Exposure.	Pharm Res.	28	712-719	2011
Hagisawa K, Nishioka T, Suzuki R, Takizawa T, Maruyama K, Takase B, Ishihara M, Kurita A, Yoshimoto N, Ohsuzu F, Kikuchi M.	Enhancement of ultrasonic thrombus imaging using novel liposomal bubbles targeting activated platelet glycoprotein IIb/IIIa complex-in vitro and in vivo study.	Int J Cardiol.	152	202-206	2011

ELSEVIER

Pathological changes in tight junctions and potential applications into therapies

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Epithelial cells are pivotal in the separation of the body from the outside environment. Orally administered drugs must pass across epithelial cell sheets, and most pathological organisms invade the body through epithelial cells. Tight junctions (TJs) are sealing complexes between adjacent epithelial cells. Modulation of TJ components is a potent strategy for increasing absorption. Inflammation often causes disruption of the TJ barrier. Molecular imaging technology has enabled elucidation of the dynamics of TJs. Molecular pathological analysis has shown the relationship between TJ components and molecular pathological conditions. In this article, we discuss TJ-targeted drug development over the past 2 years.

During evolution from single-celled to multi-celled organisms, a compartment system developed to separate the inside of the body from the outside environment. This compartment system is made up of epithelial and endothelial cell sheets. Sealing of the intercellular space between individual epithelial or endothelial cells is crucial for compartmentalization.

Tight junctions (TJs) are the apical-most component of intercellular seals. TJs are directly involved both in the sealing of paracellular spaces and in two major functions of membranes: the barrier function and the fence function [1,2]. The barrier function is the first line of defense against pathogenic microorganisms and xenobiotics, and the fence function regulates cellular polarity. Deregulation of these functions is often observed in infectious diseases, inflammation and carcinogenesis.

Freeze-fracture electron microscopy analysis has shown that TJs are a set of continuous and anastomosing strands [3]. A series of analyses revealed that TJ-seals contain integral membrane proteins, such as occludin, claudins and junctional adhesion molecules (Fig. 1) [4–6]. The claudin protein family comprises 27 members and the junctional adhesion molecule (JAM) family comprises 3 members [4,7]. A tricellular junction-sealing component, tricellulin, has also been identified in epithelial cell sheets [8]. Occludin and tricellulin contain the tetra-spanning and other related proteins for vesicle trafficking and membrane line (MARVEL) domain. Occludin and tricellulin are members of the MARVEL protein family [9]. MarvelD3, another member of the MARVEL protein family, has been identified as a component of TJs [10]. The intracellular constituents of TJs, ZO-1 and ZO-2, determine where the claudin-based strands are formed [11]. Lipolysis-stimulated lipoprotein receptors define where tricellular junctions are formed [12]. These biochemical components of TJ-seals were all clarified within a single decade [5,6,13]. Our understanding of TJ-components has provided us with a new perspective on drug delivery and drug discovery for infectious diseases, inflammations and cancers [14–16].

There have been two main progressions in our understanding of the biology of TJs within the past 2 years: mucosal barrier homeostasis and TJ barrier homeostasis. Proof-of-concepts for TJ-targeted drug delivery have been demonstrated. In this article, we discuss recent topics in TJ biology and TJ-targeted therapy.

Biology of the epithelial barrier

Tight junctions

Epithelium is central to the construction of multicellular animals. More than 60% of the cell types in the vertebrate body are epithelial cells. Epithelia enclose and partition the animal body, line all of its surfaces and cavities, and create internal compartments. Epithelial cells are structurally polarized into a basal side that is anchored to other tissue, and an apical side that is

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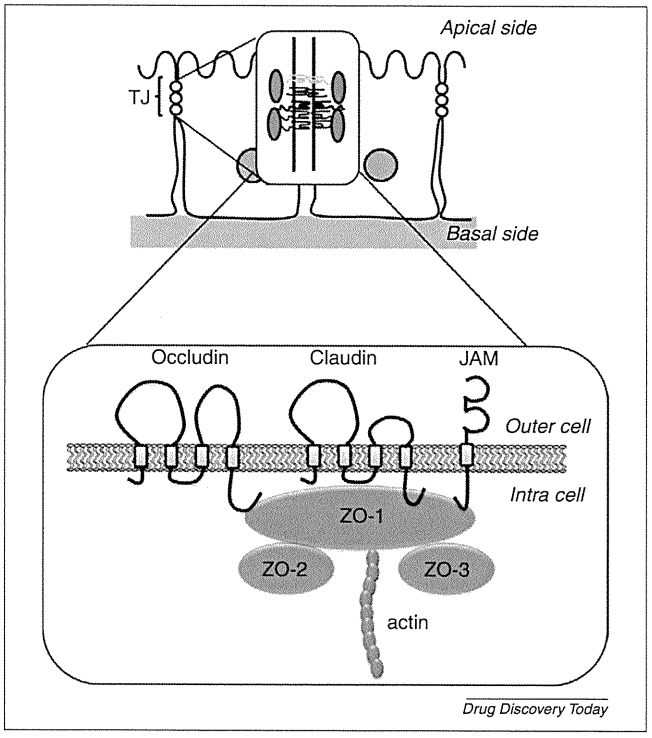

FIGURE 1

The epithelial barrier. Occludin, a tetra-transmembrane protein, was the first TJ-constituting protein identified [19]. Claudin was the second [21]. Claudins comprise a tetra-transmembrane protein family of 27 members. JAMs are glycosylated transmembrane proteins that belong to the immunoglobulin superfamily [4]. ZO-1, ZO-2 and ZO-3 are membrane-associated guanylate kinase proteins composed of a PSD95/Dlg/ZO-1 domain, an SH3 domain, a guanylate kinase domain, an acidic domain and an actin-binding region [68]. *Abbreviations*: JAMs: junctional adhesion molecules; TJ: tight junction;

unanchored. Adjacent epithelial cells are joined by occluding junctions called TJs. TJs have pivotal roles in separating the inside of the body from the outside environment, and in separating the inside and outside of tissues. TJs also function as a fence by preventing the free movement of apical membrane components and basal membrane components in epithelial cells.

TJs are intercellular sealing components located at the apical-most part of lateral membranes between adjacent epithelial cells and endothelial cells [17]. Adjacent TJ strands laterally associate with each other to form a paired strand thereby eliminating the intercellular space. Freeze fracture electron microscopy analysis revealed that TJs are continuous anastomosing intramembranous particle strands or fibrils with complementary grooves [3]. TJs are composed of transmembrane proteins, such as claudins, occludin and JAMs, in addition to cytoplasmic plaque proteins, including ZO-1, ZO-2, ZO-3 and cingulin [18].

Integral membrane proteins

Occludin was the first integral membrane protein identified in TJs [19]. Occludin has four transmembrane domains and has a molecular mass of approximately 65 kDa. Deletion of occludin does not affect the structure and function of TJs [20]. Claudins were the second integral membrane proteins identified in TJs [21]. Claudins comprise a multigene family with at least 27 members [7]. Claudins are 21–28-kDa proteins with tetratransmembrane domains. Claudins are key components in the structure and function of TJs [5,6]. A series of cellular analysis and knockout mouse analysis has clarified the roles of claudins in TJs [5,22].

Cytoplasmic proteins

ZO-1 was the first identified TJ-associated protein [23]. ZO-1, ZO-2 and ZO-3 contain PDZ-domains and the membrane-associated guanylate kinase domain. ZO-1, ZO-2 and ZO-3 are involved in formation of the TJ seal; they bind to the C-terminal cytoplasmic domain of occludin and claudins through the ZO PDZ domains [13]. ZO-1 and ZO-2 are crucial components for the definition of TJ formation [11].

Tricellular tight junctions

There are two types of TJs in epithelial cell sheets: bicellular and tricellular [2,24,25]. Occludin, claudins and JAMs are components of bicellular TJs. Tricellulin (approximately 65 kDa) is the only integral membrane component in tricellular TJs [8]. Tricellulin contains four transmembrane domains and shows structural similarity with occludin. Tricellulin is highly concentrated in tricellular TJs, but it is also localized in bicellular TJs [8,26]. Lipolysis-stimulated lipoprotein, a tricellular TJ-associated protein, defines tricellular contacts in epithelial cell sheets [12].

Mucosal barrier

The intestinal epithelium is where nutrients derived from food are absorbed, and it is also the first line of defense against microorganisms and xenobiotics. Regulation of the epithelial barrier is crucial for mucosal homeostasis. Recently, two intestinal epithelium proteins that regulate the intestinal barrier were identified.

The first protein is guanylyl cyclase C (GCC), which is a transmembrane receptor for the endogenous peptides guanylin and uroguanylin and for bacterial heat-stable enterotoxins [27]. GCC signaling has a pivotal role in the regulation of intestinal fluid and electrolyte homeostasis [28]. GCC-knockout mice show increased intestinal permeability, and GCC-knockdown in Caco-2 cells disrupts TJ integrity. This disruption of the TJ barrier is accompanied by phosphorylation of myosin II regulatory light chains, which induces TJ disassembly. GCC signaling is therefore involved in regulation of the TJ barrier [29].

The second intestinal membrane protein is matriptase. Matriptase is an integral membrane protein with trypsin-like serine protease activity and is a member of the type II transmembrane serine protease family [30]. It is widely expressed in all epithelia, and it is expressed in epithelial cells in the gastrointestinal tract [30]. Loss of matriptase reduces epithelial barrier integrity and enhances paracellular permeability. Matriptase facilitates claudin-2 loss from TJ complexes by indirect regulation of claudin-2 protein turnover by atypical protein kinase C zeta. Interestingly, matriptase does not affect some of the other TJ components, such as claudin-1, claudin-3, claudin-4, claudin-8, ZO-1, or E-cadherin [31].

These findings indicate that GCC signaling and matriptase might be potent targets for the treatment of intestinal disorders whose pathogenesis is disruption of the intestinal barrier function leading to mucosal inflammation and immune activation.

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TJ dynamics

TJs are complexes of transmembrane and peripheral membrane proteins, including occludin, claudins, ZO-1 and ZO-2 [6]. The TJ structure is highly dynamic and undergoes continuous remodeling through unique kinetics [32]. The properties of TJs are determined by these dynamics [33].

Occludin S408 dephosphorylation reduces paracellular cation influx by stabilizing the occludin–ZO-1 interaction, leading to enhancement of claudin-1 and claudin-2 exchange and reduction of their pore formation at the TJ. By contrast, occludin S408 phosphorylation enhances homotypic occludin–occludin interactions, leading to the release of ZO-1 and formation of claudin-1- and claudin-2-based pores. Therefore, occludin S408 phosphorylation is a key factor in the remodeling of the claudin–occludin–ZO-1 interaction [34].

Claudin-1 is stably localized in TJs [35]. Most occludin is mobile and diffused within the junctional membrane. By contrast, most ZO-1 is continuously exchanged between the membrane and cytosol pools [34]. Fluorescence recovery after photo-bleaching (FRAP) analysis provided new insights into the dynamics of TJs. The perijunctional actomyosin ring contributes to myosin light chain kinase (MLCK)-dependent TJ regulation. FRAP analysis showed that TJ-associated ZO-1 exists in three pools: a fixed pool, a fast exchangeable pool associated with the cytosolic pool, and a slow exchangeable pool associated with the cytosolic pool. The exchange between the TJ pools and the cytosolic pool is regulated by MLCK [36]. Claudin dynamics differ depending on the particular claudin. Claudins forming TJ strands showed slower dynamics than those not forming TJ strands. Distinct claudin stabilities might affect how TJs regulate paracellular permeability by altering paracellular flux and paracellular ion permeability [37].

These insights into the dynamics of TJs address the molecular mechanism of paracellular homeostasis and will hopefully lead to the development of TJ-targeted tissue-specific and solute-specific drug delivery systems.

Epithelial barrier as the first line of defense against pathological microorganisms

The human mucosa has a surface area equivalent to 1.5 tennis courts. This large surface area means that there is significant risk of infection by pathological microorganisms; therefore, homeostasis of the epithelial barrier is important. Indeed, some pathogens modulate the epithelial barrier to facilitate easy and widespread infection (Fig. 2a).

Modulation of the epithelial barrier by pathogens

Human immunodeficiency virus-1 (HIV-1) infection is often associated with increased permeability of mucosal epithelial cells. Viral envelope glycoprotein (gp)120 is a crucial viral protein that increases the permeability of the epithelial barrier. When HIV-1 binds to cells it induces production of TNF-α, leading to a decrease in mucosal epithelial barrier integrity and spread of HIV-1 infection [38].

Atopic dermatitis (AD) is the most common inflammatory skin disease [39], and susceptibility to cutaneous infections is increased in AD patients. Widespread skin infection by the herpes simplex virus (HSV) causes severe viral complications, such as eczema herpeticum in AD patients. Defects in the epidermal TJ barrier increase the susceptibility of patients with AD to widespread subcutaneous infection with HSV or other viral pathogens [40]. In the early stage of infection with enterohemorrhagic *Escherichia coli* (EHEC), non-bloody diarrhea occurs in the absence of shiga toxin. EHEC infection increases expression of claudin-2 and redistribution of claudin-3 and occludin. These changes correlate with increased intestinal permeability [41]. Infection by enteroaggregative *Escherichia coli* (EAEC) causes dissociation of claudin-1 from the TJs between epithelial cells, leading to disruption of the TJ barrier [42]. By contrast, respiratory syncytial virus (RSV) increases TJ integrity. RSV is the major cause of bronchitis, asthma and severe lower respiratory tract diseases in infants and young children [43]. RSV infection induces expression of claudin-4 and occludin in human nasal epithelial cells. Induction of TJ components has a crucial role in epithelial cellular polarity, leading to budding of the virus from the epithelial apical surface [44]. Therefore, prevention of TJ barrier modulation by pathogens might be a viable therapeutic strategy.

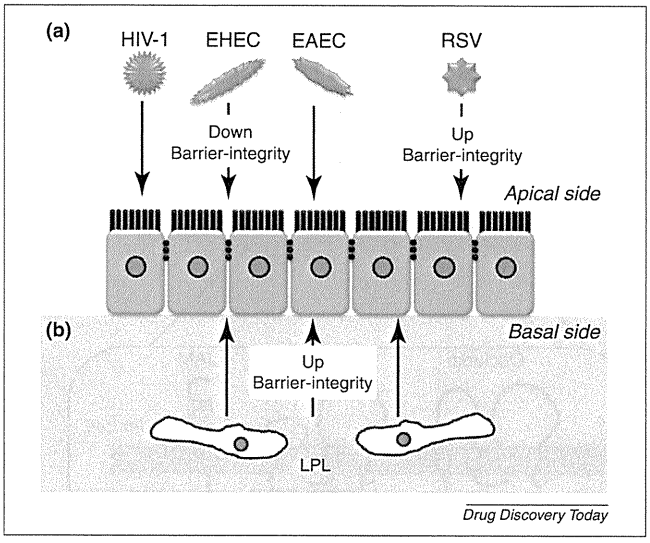

FIGURE 2

Regulation of the first line of defense, the epithelial barrier. **(a)** Pathological microorganism–epithelial barrier interaction. Infection of epithelial cells by HIV-1, EHEC, or EAEC decreased epithelial barrier integrity [38,41,42]. By contrast, RSV infection increased the barrier function [44]. **(b)** Lymphocyte–epithelial barrier interaction. LPLs regulate the integrity of the epithelial barrier via direct interaction with epithelial cells through notch signaling [49]. *Abbreviations*: EAEC: enteroaggregative *Escherichia coli*; EHEC: enterohemorrhagic *Escherichia coli*; HIV-1: human immunodeficiency virus-1; LPLs: lamina propria lymphocytes; RSV: respiratory syncytial virus.

Lymphoepithelial cross talk in the epithelial barrier

Mucosa-associated lymphoid tissues (MALTs) are lymphoid immune tissues that are located in the mucosal epithelium. By activating mucosal immune responses, they function as the first line of defense against pathogens invading the body through the epithelium [45]. MALTs comprise gut-associated lymphoid tissues, nasopharynx-associated lymphoid tissues and bronchus-associated lymphoid tissues. MALTs contain lymphocytes, M cells, T cells, B cells and antigen-presenting cells. Recently, lamina propria lymphocytes (LPLs) underlying the intestinal epithelium have

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been shown to have a crucial role in the homeostasis of the epithelial barrier (Fig. 2b). Direct interaction of LPLs with intestinal epithelial cells is essential for the barrier function of the intestinal epithelium [46]. The notch signaling pathway regulates cell fate decisions through cell–cell interactions [47]. Notch signaling determines the differentiation of intestinal stem cells into secretory cells, absorptive cells, or enterocytes [47,48]. The absence of LPLs in mice causes increased intestinal permeability and a lack of activation of notch in colonocytes [49]. Transfer of LPLs to LPL-deficient mice decreased intestinal permeability and activated notch signaling in colonocytes. In Caco-2 cells, knockdown of notch mRNA reduced the epithelial barrier function, and was accompanied by upregulation of claudin-2 proteins, reduction of occludin and cytoplasmic localization of claudin-5 [49]. Therefore, lymphoepithelial cross talk might regulate epithelial differentiation and barrier integrity. Notch signaling is highly activated in the mucosa of patients with Crohn's disease, leading to dysregulation of the differentiation of epithelial cells [49]. Normalization of disruption of this cross talk might be a potent strategy for treating immune-mediated intestinal disorders.

Proof-of-concept for TJ-targeted drug development

As mentioned in the introduction, epithelial cells are a potent target for drug development. TJ-targeted drug development has been attempted [14,50], and proof-of-concepts for TJ-targeted drug absorption, cancer targeting and mucosal vaccination have been established. Recent findings indicate that TJ-targeted therapy for hepatitis C virus (HCV), diabetes and inflammatory diseases might be possible.

HCV infection

A total of 170 million people worldwide are infected with the HCV. Hepatitis C is the leading cause of chronic liver inflammation, cirrhosis and cancer. Claudin-1 and occludin are co-receptors for HCV infection, indicating that binders to claudin-1 or occludin might be potent inhibitors of HCV entry [16]. DNA immunization enabled successful preparation of monoclonal anti-claudin-1 antibodies against the extracellular loop of claudin-1, and these anti-claudin-1 antibodies prevented HCV infection. Antibodies effectively blocked cell entry of highly infectious escape variants of HCV that were resistant to neutralizing antibodies [51]. When hepatitis C patients reach end-stage liver failure, liver transplantation is the only choice for curative treatment; however, reinfection of the transplanted liver by HCV often occurs. There is a significant correlation between hepatic levels of claudin-1 and occludin and HCV reinfection after liver transplantation [52]. Inhibition of HCV reinfection of the transplanted liver by using anti-claudin-1 antibodies might be a potent treatment for patients with liver transplantation.

Diabetic retinopathy

Breakdown of the blood–retinal barrier (BRB) is a hallmark of diabetic retinopathy [53]. Alterations to the BRB occur early in the progression of diabetic retinopathy and eventually lead to macular edema, which is responsible for vision loss [54]. Diabetic patients show elevated levels of TNF-α in the vitreous humor. TNF-α increases the permeability of retinal endothelial cells. TNF-α decreases ZO-1 and claudin-5 expression and alters cellular localization of ZO-1 and claudin-5 [55]. Thus, regulation of BRB-integrity might be a potent strategy for treating vision loss owing to diabetes. Indeed, a chemical already in clinical use for the treatment of diabetic retinopathy, calcium dobesilate, attenuates the decrease in occludin and claudin-5 and prevents BRB breakdown [56]. Berberine, a plant alkaloid, has also been used for the treatment of diabetes. Berberine prevents barrier defects in retinal epithelial cells [57]. Inducers of occludin and claudin-5 or promoters of TJ integrity could be a potent treatment for diabetic retinopathy.

Inflammatory diseases

Berberine has been also used in the treatment of gastroenteritis and diarrhea. TNF-α disrupts TJ integrity in inflammatory bowel diseases (IBD). Regulation of the TNF-α-dependent signaling pathway is a potent strategy for the treatment of IBD. TNF-α removes claudin-1 from TJs and induces claudin-2 expression, leading to disruption of the TJ barrier. Attenuation of TNF-α signaling is a potent strategy for IBD therapy. Berberine also attenuates TNF-α-induced TJ barrier defects by removing claudin-1 and inducing claudin-2 expression [58]. Spontaneous colitis was observed in interleukin (IL)-10−/− mice in which paracellular permeability was increased in conjunction with decreased expression and redistribution of ZO-1, occludin and claudin-1. Treatment with a probiotic, *Lactobacillus plantarum*, restored expression of TJ components and TJ integrity, resulting in prevention of bacterial translocation and proinflammatory responses in IL-10−/− mice [59]. Recovery of TJ integrity might be a potent strategy for inflammatory intestinal diseases. Ouabain, which is an inhibitor of Na^+, K^+-ATPase, increased TJ integrity through signaling pathways involving c-Src and ERK1/2 and by modulating the expression of claudin-1, claudin-2 and claudin-4 [60,61]. Several natural products have been found to be therapeutically useful against epithelial barrier defects.

Paracellular drug transport

The claudin protein family comprises 27 members [7]. Claudins form homo- and hetero-type strands in the lateral membrane. Adjacent claudin-based TJ strands associate with each other, leading to sealing of the intercellular space. The combination of the claudin members is a determinant factor for the properties of the TJ barrier [5]. These findings suggest that optimization of claudin modulators with narrow-specificity in certain cases, or broad-specificity in other cases, might regulate solute- and tissue-specificity in paracellular transport. The most important issue in TJ-targeted drug absorption is the development of claudin modulators. Claudin is an integral membrane protein with a tetra-transmembrane domain. Claudin binders are the first choice for claudin modulators. The first extracellular loop contains approximately 50 amino acids and the second contains approximately ten amino acids. Claudins are hydrophobic proteins, and preparation of a recombinant protein is only currently possible for claudin-4 [62]. Therefore, the development of claudin binders, including antibodies, has been slow. Budded baculoviruses display functional forms of membrane proteins on their surface [63]. Claudin-displaying budded baculoviruses possess a native form of claudin and can be used as a screening system for claudin binders [64]. Functional membrane proteins are heterogeneously expressed on

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budded baculoviruses [63]. Functional information using FRAP analysis will enable development of a screening system for claudin modulators with narrow- or broad-specificity using the heterogenous claudin-displaying baculoviral system. We predict that, in the near future, proof-of-concept for tissue- and solute-specific paracellular transport by modulating the claudin-barrier will be demonstrated.

Coupling of transcellular and paracellular transport systems controls permeability to solutes [65]. Claudin-based TJs function as charge-selective paracellular channels [6]. Claudin-15 is responsible for transepithelial permeability to extracellular monovalent cations, especially Na^+. Claudin-15-deficient mice exhibit low luminal Na^+ levels and low glucose absorption in the intestine, indicating that paracellular transport of Na^+ through claudin-15-based TJ strands might be coupled to transcellular transport of glucose through a glucose transporter [66]. These findings suggest that modulation of the claudin-mediated paracellular transport of solutes might regulate the transcellular transport of drugs through a transporter.

Concluding remarks

To our knowledge, the first report of TJ-targeted drug development was the discovery in 1961 of enhanced mucosal absorption of drugs by co-administration of ethylenediaminetetraacetic acid [67]. TJs were identified in 1963 [17]. Modulation of the TJ-barrier has been a major strategy for enhancing mucosal absorption; however, the biochemical structure of TJs was unclear until 1998. Until that year, absorption enhancers were screened mainly by modulating epithelial cell sheets. Recent imaging studies have begun to reveal the dynamics of TJs and also how these dynamics are regulated [36,37]. Future detailed analyses using FRAP will provide us with new insights into strategies for modulation of the TJ barrier. In addition to TJ-modulated drug absorption, TJ-targeted therapy for HCV infection and diabetic retinopathy has recently been proved effective [51,56]. The questions of how TJ dynamics are regulated, and how expression of TJ components is regulated are still to be answered. The molecular pathology of deregulation of the TJ barrier is not yet fully understood. TJ-targeted drug development has been spearheaded by rapid progress in our understanding of the biology of the TJ barrier.

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