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Mucosal vaccination using claudin-4-targeting

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ABSTRACT

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

1. Introduction

Each year, 17 million people die from infectious diseases worldwide, and 7 million people die from cancers worldwide (http://www.globalhealth.org/infectious_diseases/; http://www.reuters.com/article/healthNews/idUSN1633064920071217). Thus, the development of methods to prevent and treat infectious diseases and cancers is an important issue for healthcare worldwide. Vaccination against these diseases is a promising approach because of its low frequency of side effects and its great preventative and therapeutic effects. Vaccination strategies are classified as parenteral or mucosal. Parenteral vaccination is effective for the elimination of infectious cells and cancer cells by the induction of systemic immune responses. Parenteral vaccines are administered by injections, which are invasive, painful, and have low levels of patient compliance; moreover, mucosal immunological defense is not induced. In contrast, mucosal vaccine elicits both mucosal and systemic immune responses, resulting in the prevention of infection on the mucosal surfaces and the elimination of pathological cells [1–3]. Mucosal administration is needle-free, less painful, and has improved patience compliance. Thus, mucosal vaccination appears to be an ideal vaccination strategy, although mucosally administered protein antigens are poorly immunogenic. Various approaches for the mucosal delivery of antigens have been investigated [4–6]. Mucosa-associated lymphoid tissues (MALTs) play pivotal roles in mucosal immunological responses [7,8]. MALTs comprise gut-associated lymphoid tissues (GALT), nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT). MALT contains lymphocytes, M cells, T cells, B cells and antigen-presenting cells (APCs), and the efficient delivery of antigens into MALT is essential for mucosal vaccinations [9]. Indeed, there have been several attempts to deliver antigens to MALT using microparticles, liposomes, saponins or chitosans [4–6].

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

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

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

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

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

2. Materials and methods

2.1. Animals

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

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

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

2.3. Immunoblotting for claudin-4

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

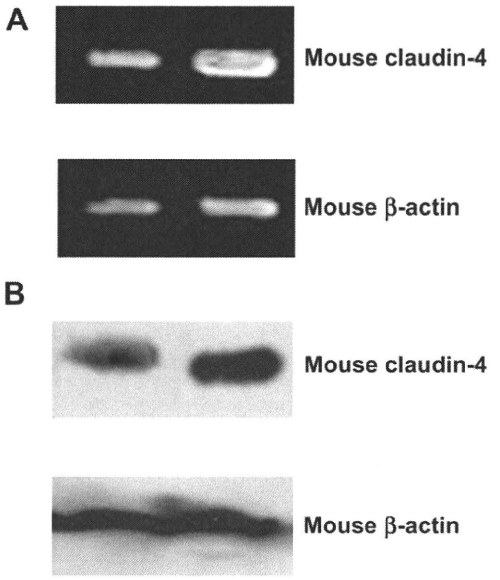

Fig. 1. Expression of claudin-4 in NALT. A) RT-PCR analysis. mRNA was isolated from NALT of mice, and expression of claudin-4 was assayed by RT-PCR. B) Immunoblot analysis. The lysate of NALT was subjected to SDS-PAGE, followed by western blotting with anti-claudin-4 Ab. β-actin was used as an internal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

2.4. Preparation of OVA-C-CPE fusion proteins

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

2.5. Enzyme-linked immunosorbent assay (ELISA)

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

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

2.6. Nasal immunization

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

2.7. OVA-specific antibody production

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

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

2.8. Cytokine ELISA

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

2.9. Cell cultures

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

2.10. Anti-tumor activity

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

2.11. Statistical analysis

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

3. Results

3.1. Expression of claudin-4 in NALT

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

3.2. Preparation of claudin-4-targeting OVA

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

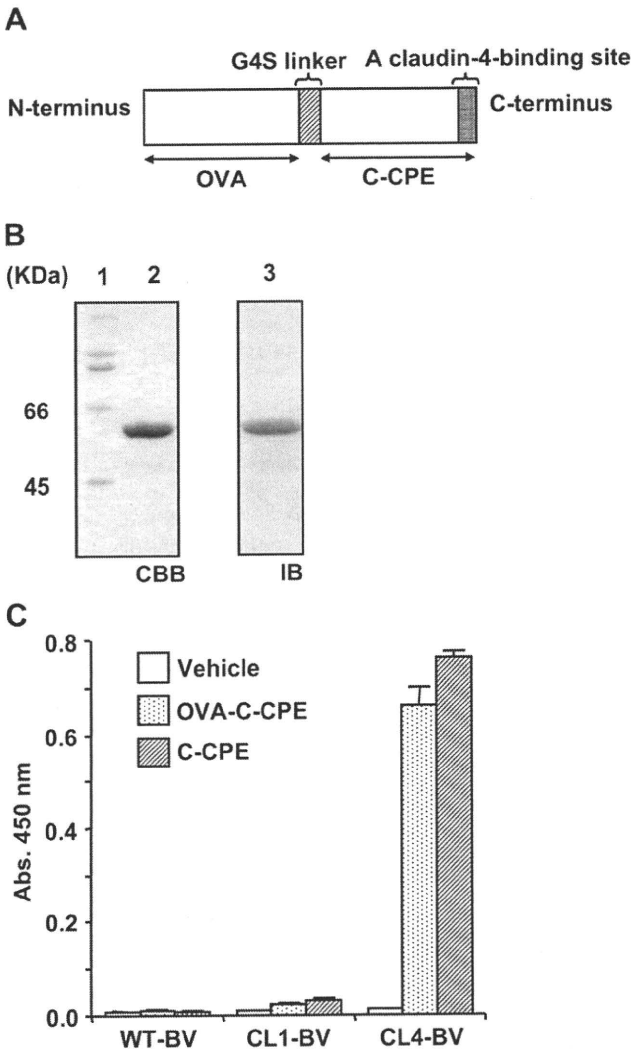

Fig. 2. Preparation of OVA-C-CPE. A) Schematic illustration of OVA-C-CPE. The claudin-4-binding site of C-CPE is located in the C-terminal 16 amino acids [29]. OVA was fused with C-CPE at the N-terminal of C-CPE, resulting in OVA-C-CPE. B) Purification of OVA-C-CPE. OVA-C-CPE was expressed in *E. Coli*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

3.3. Induction of OVA-specific humoral responses

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

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

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

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

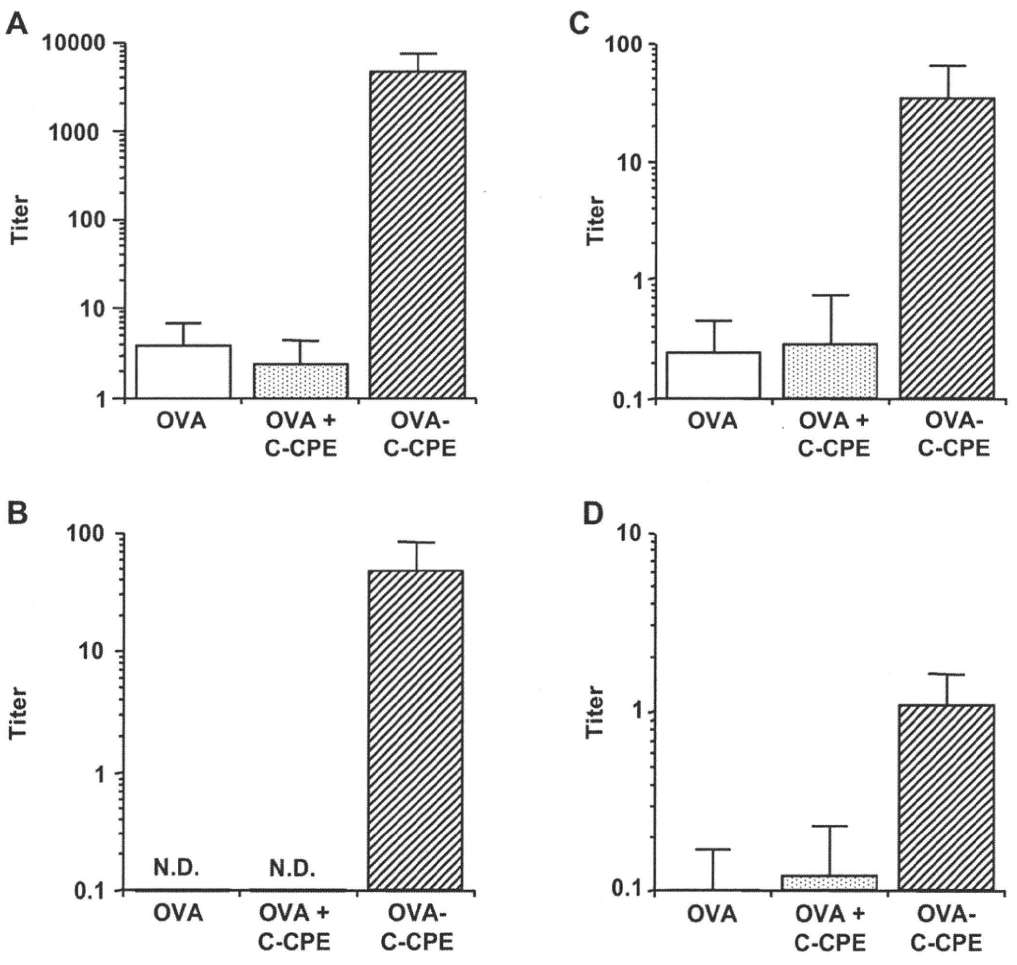

Fig. 3. Production of OVA-specific IgG and IgA by OVA-C-CPE. Mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, or OVA-C-CPE (5 μg OVA) once a week for 3 weeks. Seven days after the last immunization, the levels of serum IgG (A), nasal IgA (B), vaginal IgA (C) and fecal IgA (D)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

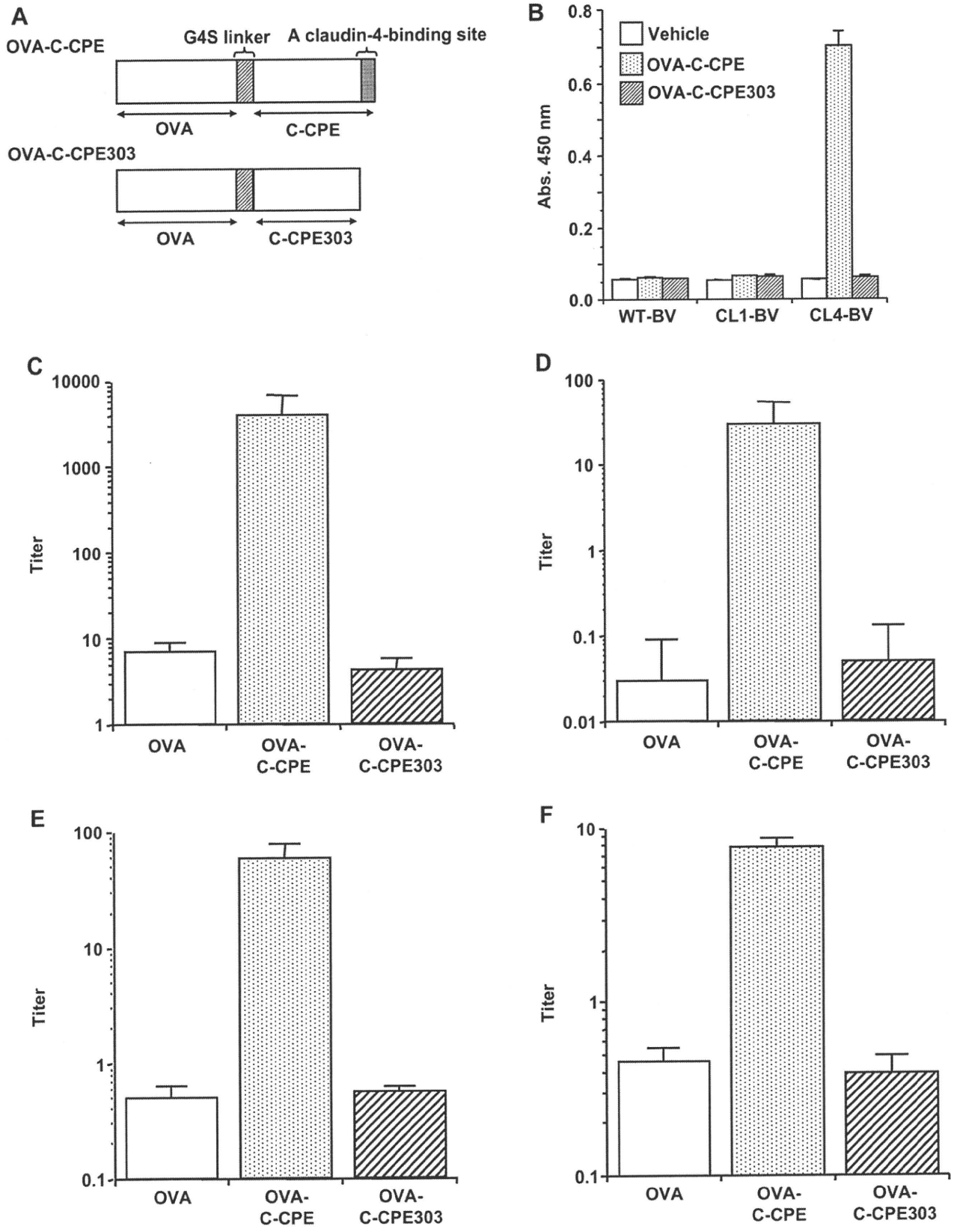

Fig. 4. Involvement of claudin-4 in the immune responses to OVA-C-CPE. A) Schematic illustration of OVA-C-CPE mutant. The C-terminal 16 amino acid-deleted C-CPE mutant (C-CPE303) did not bind to claudin-4 [29]. To clarify the involvement of claudin-4 in the immune response initiated by OVA-C-CPE, OVA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

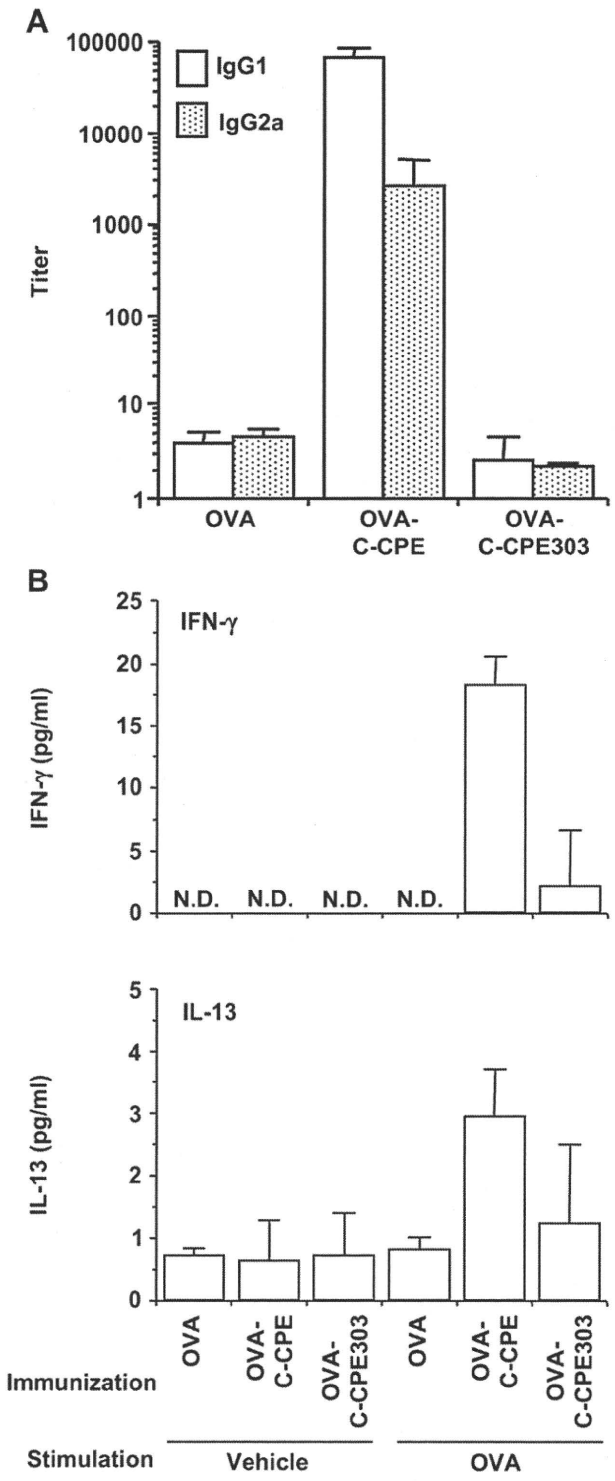

Fig. 5. Th1 and Th2 responses induced by OVA-C-CPE. Mice were nasally immunized with OVA, OVA-C-CPE or OVA-C-CPE303 (5 μg of OVA) once a week for 3 weeks. Seven days after the last immunization, serum and splenocytes were collected. Serum IgG subclass (IgG1 and IgG2a) was determined by ELISA (A). The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

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

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

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

4. Discussion

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

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

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

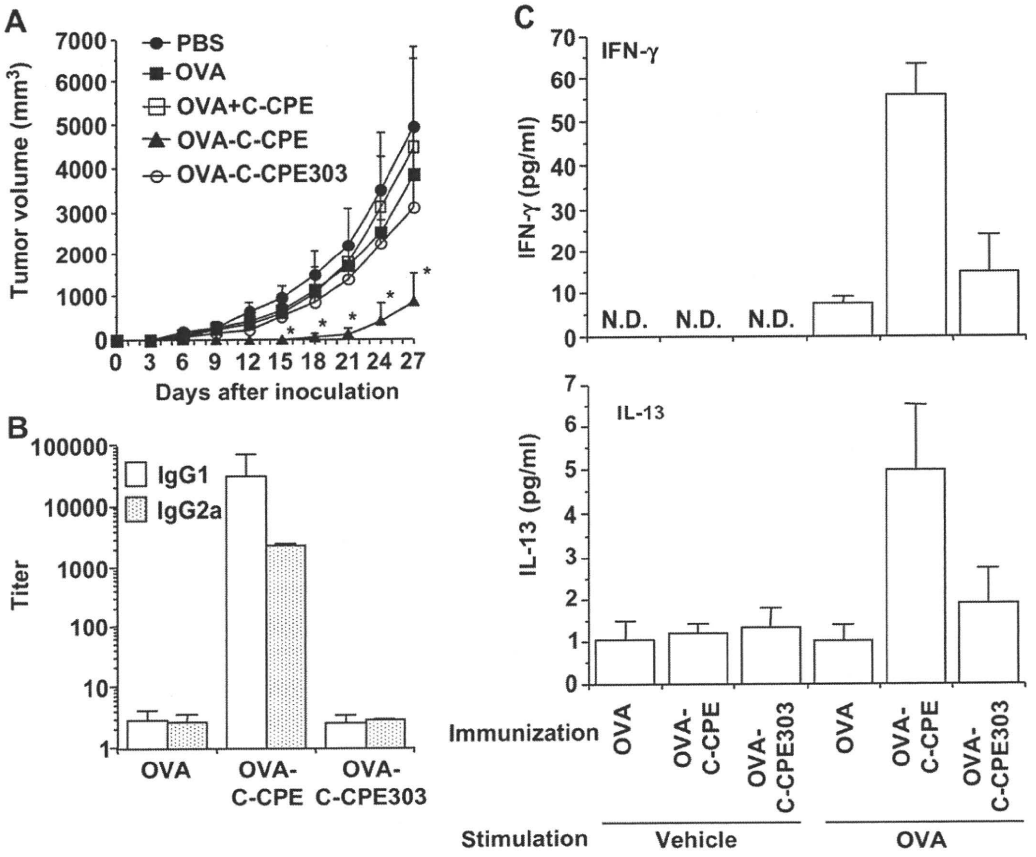

Fig. 6. Anti-tumor activity induced by immunization with OVA-C-CPE in an EG7 cancer model. A) Protective immune response against tumor challenge. C57BL/6 mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA-C-CPE, or OVA-C-CPE303 (5 μg of OVA) once a week for 3 weeks. Seven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

[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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—Reviews—

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 maintianing 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潅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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本総説は，日本薬学会第129年会シンポジウム S24 で発表したものを中心に記述したものである．

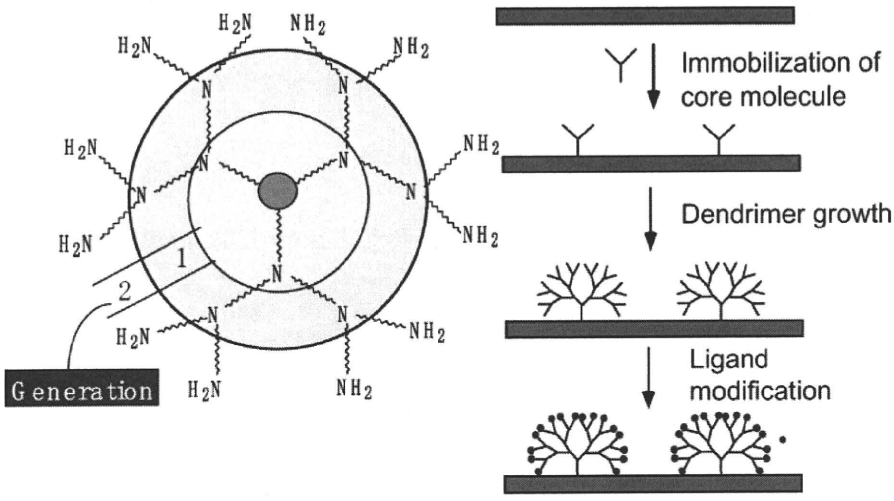

Fig. 1. Structure of Polyamidoamine Dendrimer and Application for Cell Culture

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，細胞が高機能化す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

大阪大学大学院薬学研究科教授．1981 年大阪大学大学院博士後期課程修了（薬学博士）．1982–1984 年米国メリーランド大薬学部，NIH（NIEHS）で博士研究員として勤務．1983 年大阪大学薬学部助手．1992 年同助教授，2000 年 3 月より現職．現在 C 型肝炎など肝疾患を対象とした創薬研究に取り組んでいる．

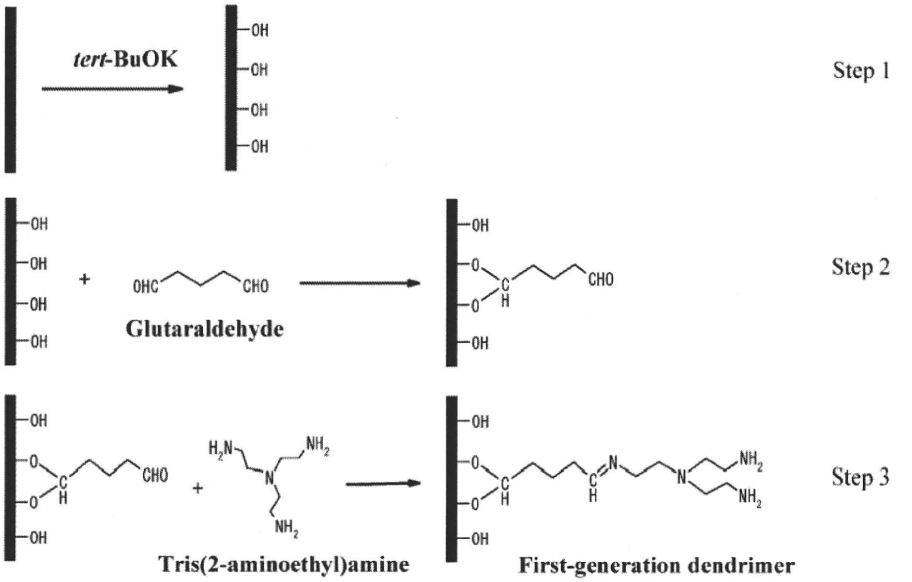

Fig. 2. Schematic Illustration of Dendrimer Immobilization onto the Surface of Polystyrene Plate

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い出している．[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アクター（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，潅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潅流培養を行った．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．プラスティ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

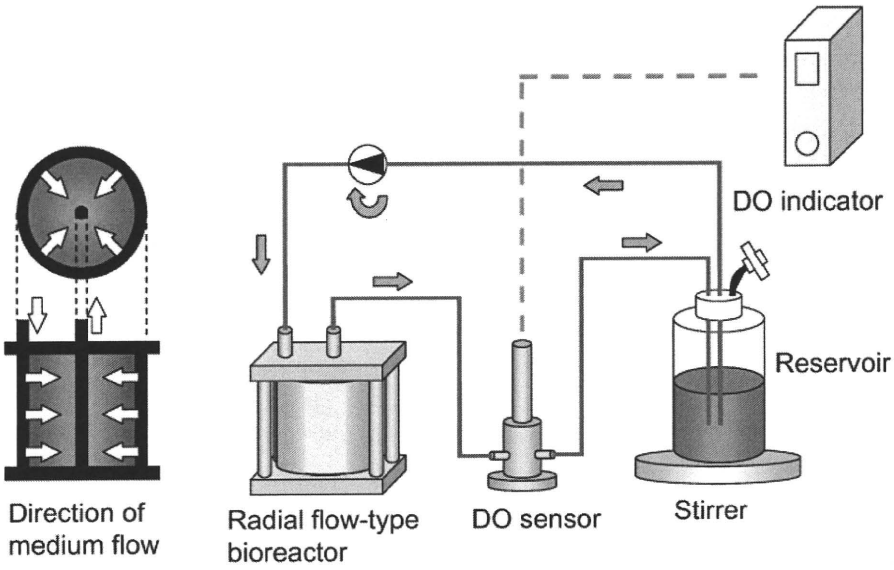

Fig. 3. Perfusion Culture of Hepatocytes by Radial Flow-type Bioreacor

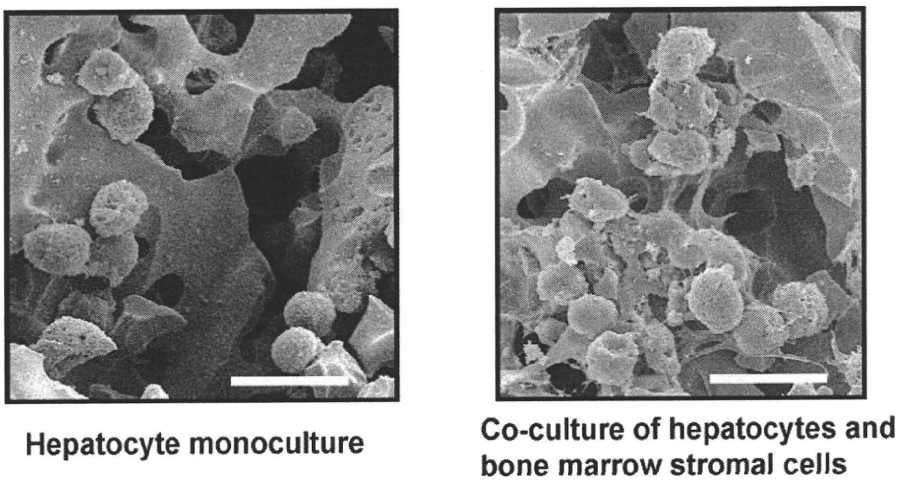

Fig. 4. Electron Micrographs of Hepatocytes Cultured in Silan Beads
White bar indicates 50 μm.

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に肝細胞を播種した．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 日間の連続潅流培養を行った．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 による潅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的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出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替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替わりに生理食塩水を門脈から投与したもの (sham operation) を用意した．凍結肝臓切片を作成し蛍光観察を行った．細胞移植群においては誘導培地，非誘導培地で培養した双方で生着が確認された．骨髄由来の間葉系幹細胞 (BMSC) を移植した際には個々の蛍光が散在していたが，[22] 歯胚由来細胞移植の場合はコロニー状の像が観察されたことから生着後に増殖したものと思われる．*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癒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バイオ人工肝臓へ利用することができること，医薬品スクリーニングの評価系としても，倫理的な問題を持つ ES 細胞，強制的に未分化状態に回帰させた iPS 細胞由来のものに比べ有用であると考えている．

6. おわりに

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

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

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ABSTRACT

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

1. Introduction

Chemotherapeutic agents target the intracellular metabolic processes or growth rates that are different between malignant cells and normal cells, and rapidly growing cancer cells are sensitive to chemotherapies [1,2]. But, progressive cancer cells with a decreased growth rate respond poorly to chemotherapy [3]. Radiation therapy affects both the tumor and the surrounding normal tissue. These conventional therapies cause DNA damage, leading to genomic instability and susceptibility to neoplastic mutations [4]. Cancer cells often overexpress surface proteins, including growth factor receptors or antigens [5]; thus, targeting cancer cells by using the surface proteins is a promising strategy for cancer therapy. Ligands for growth factor receptors and cytokine receptors have been fused with fragments of bacterial toxins, such as *Pseudomonas* exotoxin and *diphtheria* toxin (DT) [3,6].

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

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

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

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

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

2. Materials and methods

2.1. Chemicals

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

2.2. Cell culture

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

2.3. Preparation of DTA–C-CPE

DTA (CRM45) cDNA was kindly provided by Dr. K. Kohno (Nara Institute of Science and Technology, Japan) [21]. The plasmids containing DTA fused with C-CPE were prepared as follows. DTA was amplified by polymerase chain reaction (PCR) with pTA–DTA as a template, a forward primer (5′-GCGGTACCATGGGCGCTGAT GATGTTGTTG-3′, *Kpn*I site is underlined) and a reverse primer (5′-CCTTAATTAATCGCCGTACGCGATTTCCTG-3′, *Pac*I site is underlined). The resulting PCR fragments were subcloned into *Kpn*I/*Pac*I-digested pETH$_{10}$PER (kindly provided by Dr. Y. Horiguchi, Osaka University, Japan), and the sequence was confirmed (pET–DTA–C-CPE). Double-stranded oligonucleotide of G/S linker was prepared by annealing (heating at 95 °C for 5 min and chilling at room temperature for 60 min) of single-strand oligonucleotides, a forward oligonucleotide (5′-TGGAGGAGGAGGATCTGGAGGAGGAGGA TCTGGAGGATACCCATACGACGTCCCAGACTACGCTAT-3′, *Pac*I site is underlined) and a reverse oligonucleotide (5′-AGCGTAGTCTGGGA CGTCGTATGGGTATCCTCCAGATCCTCCTCCTCCAGATCCTCCTCCTCCA AT-3′, *Pac*I site is underlined). The resulting oligonucleotides were subcloned into *Pac*I-digested pET–DTA–C-CPE, and the sequence was confirmed (pET–DTA-linker-C-CPE).

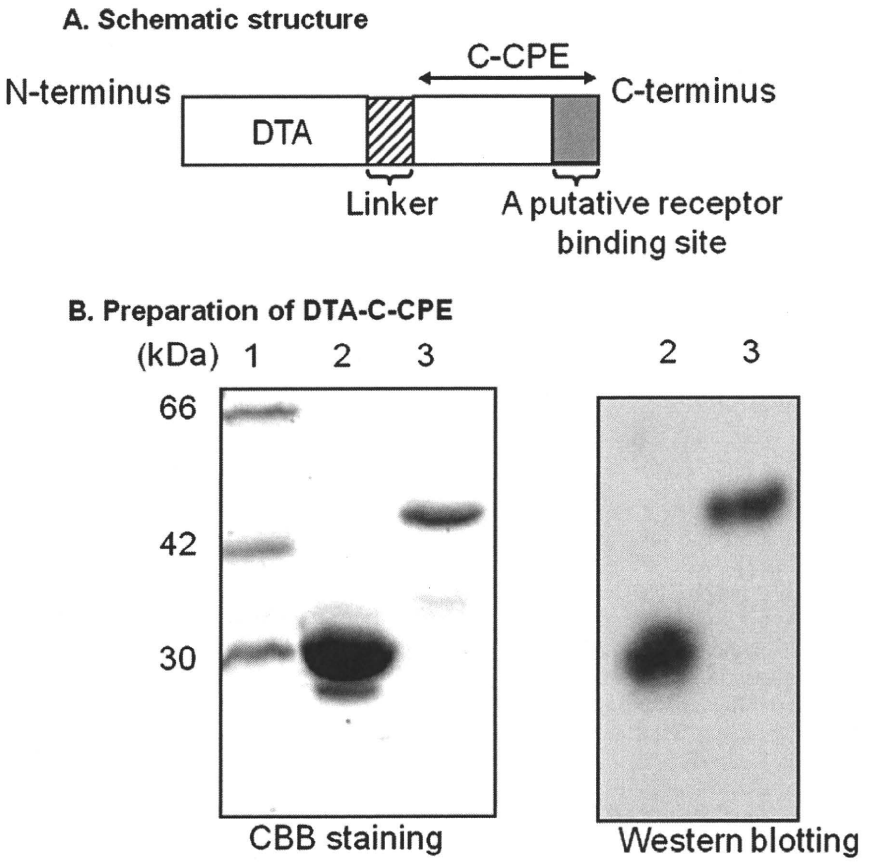

Fig. 1. Preparation of DTA–C-CPE. (A) Schematic structure of DTA–C-CPE. DTA–C-CPE is a fusion protein of DTA and C-CPE with a linker indicated by a slashed column. A dark column indicates a putative receptor-binding region of C-CPE. (B) Preparation of DTA–C-CPE. DTA or DTA–C-CPE was produced by a conventional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

The plasmid, pET–DTA-linker-C-CPE, was transduced into *Escherichia coli* strain BL21 (DE3), after which the cells were cultured in LB medium supplemented with 100 μg/ml ampicillin at 37 °C until the logarithmic phase. Isopropyl-D-thiogalactopyranoside (0.25 mM) was added to the medium, and the cells were cultured for an additional 3 h. The cells were harvested and then lysed in buffer A (10 mM Tris–HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol). The lysates were centrifuged, and the resultant supernatant was applied to HiTrap Chelating HP (GE Healthcare, Little Chalfont, UK). DTA–C-CPE was eluted by buffer A containing imidazole. The solvent was exchanged with phosphate-buffered saline by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80 °C until use. Purification of DTA–C-CPE was confirmed by sodium dodecylsulfate polyacrylamide gel electrophoresis, followed by staining with Coomassie Brilliant Blue and immunoblotting with anti-His-tag antibody. Protein was quantified by using a protein assay kit (Pierce Chemical, Rockford, IL) with BSA as a standard.

2.4. Cytotoxic activity

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

2.5. Competition assay

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

3. Results

3.1. Preparation of DTA–C-CPE

When DTA enters the cytosol, it inhibits elongation factor 2 through ADP-ribosylation and induces the inhibition of protein synthesis, leading to cell death [20,22]. C-CPE is a receptor-binding domain of CPE, and the CL-4-binding region is located on the C-terminal of C-CPE [23]. To prepare a CL-4-targeting molecule, we genetically fused DTA with C-CPE at the N-terminal of C-CPE and C-terminal of DTA. A schematic illustration of DTA–C-CPE is shown in Fig. 1A. DTA–C-CPE was produced in *E. coli* and was purified by affinity chromatography with Ni-resins. The molecular size of DTA–C-CPE, as determined by SDS–PAGE and immunoblotting, was identical to its putative size (43.2 kDa, Fig. 1B).

3.2. Cytotoxic properties of DTA–C-CPE

To examine the cytotoxicity of DTA–C-CPE, we investigated the effects of DTA–C-CPE on CL-4-expressing L (CL4/L) cells. DTA had no effect on CL4/L cells at 20 μg/ml, whereas DTA–C-CPE dose-dependently decreased the viability, reaching 39.7% relative viability at 1 μg/ml (Fig. 2A). Similar results were observed in the LDH-release assay. As shown in Fig. 2B, 5 μg/ml of DTA did not cause a release of cellular LDH; but, DTA–C-CPE at 5 μg/ml significantly increased the release of cellular LDH from 4.7% to 25.0%.

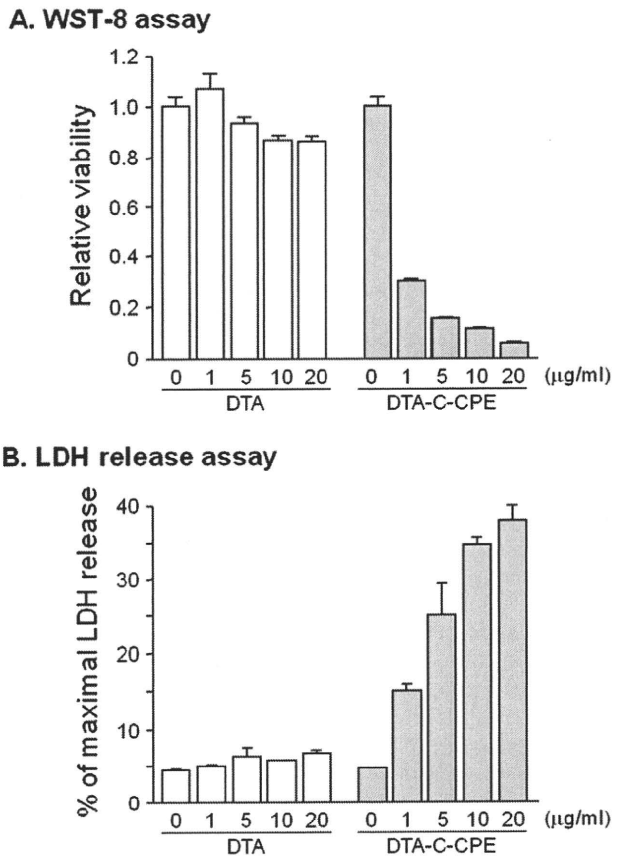

Fig. 2. Cytotoxicity of DTA–C-CPE. CL4/L cells were treated with DTA or DTA–C-CPE at the indicated concentration for 48 h. The cellular viability was measured by WST-8 assay (A) or LDH-release assay (B). Data are the mean ± SD ($n = 3$). The data are representative of three independent experiments.

3.3. Targeting properties of DTA–C-CPE

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

4. Discussion

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

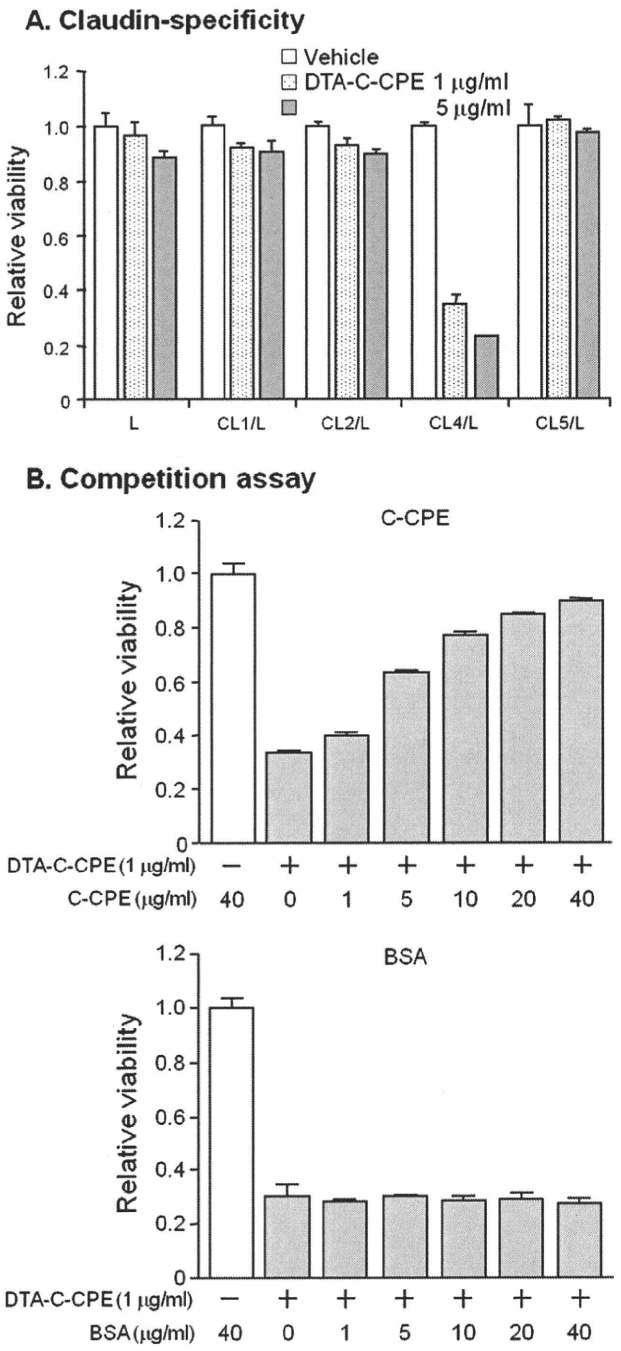

Fig. 3. Cytotoxic properties of DTA–C-CPE. (A) Claudin-specificity. L, CL1/L, CL2/L, CL4/L or CL5/L cells were treated with DTA–C-CPE at the indicated concentration for 48 h. After incubation, the cellular viability was measured by WST-8 assay. Data are the mean ± SD ($n = 3$). The data are representative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

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

DTA kills cells by inactivating elongation factor 2 when one molecule of this protein is introduced into the cytosol [24]. DTA permits the successful targeting of cells displaying only a limited number of tumor-specific growth factor receptors or antigens overexpressed on their surface, and immunotoxins containing DTA, ONTAK and DT 388GMCSF are used clinically for cancer-targeted therapy [25–27]. Therefore, we selected DTA as a cytotoxic molecule for the present study.

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

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

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