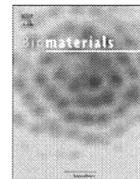


Splicing Regulation from Pro- to Anti-angiogenic VEGF Isoforms

the same retinal angiogenesis model. These data suggest that anti-SRPK1 inhibitors may be useful anti-angiogenic agents, suggesting a use in cancer as well as diabetic retinopathy or age-related macular degeneration.

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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.

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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.

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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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 patient 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

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'-ggttgtagaagtcgcccgatg-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'-ggcgtgatggtggcctgg-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 μ g 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).

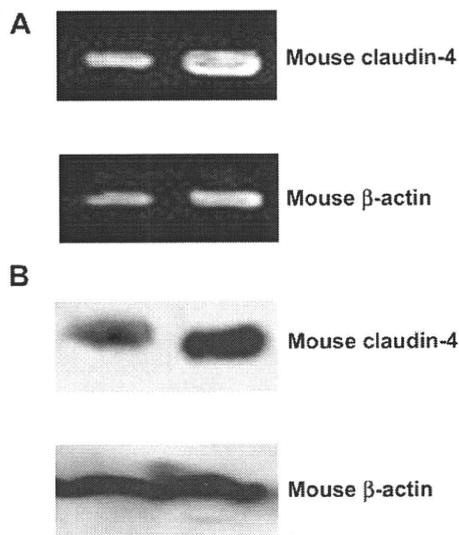


Fig. 1. Expression of claudin-4 in NALT. A) RT-PCR analysis. mRNA was isolated from NALT of mice, and expression of claudin-4 was assayed by RT-PCR. B) Immunoblot analysis. The lysate of NALT was subjected to SDS-PAGE, followed by western blotting with anti-claudin-4 Ab. β -actin was used as an internal 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'-gcggtaccatggctccatcgccgagc-3', KpnI site is underlined), and a reverse primer (5'-ccttaattaaggggaaacacatctgccaa-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- β -thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris-HCl, pH 8.0, 400 mM NaCl, 5 mM MgCl₂, 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- μ l 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 μ g of OVA and 1.89 μ g 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 \times g for 10 min, and the resultant supernatants were used as fecal extracts. Vaginal and nasal mucosa were washed with 100 or 200 μ l 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 μ g/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 μ g/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 μ g). 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).

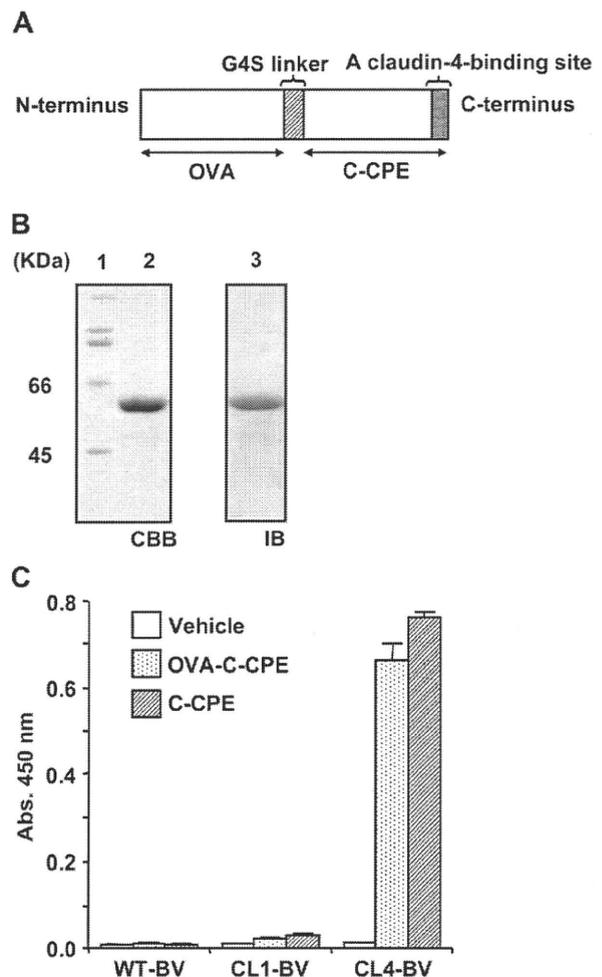


Fig. 2. Preparation of OVA-C-CPE. A) Schematic illustration of OVA-C-CPE. The claudin-4-binding site of C-CPE is located in the C-terminal 16 amino acids [29]. OVA was fused with C-CPE at the N-terminal of C-CPE, resulting in OVA-C-CPE. B) Purification of OVA-C-CPE. OVA-C-CPE was expressed in *E. coli* 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 \pm 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

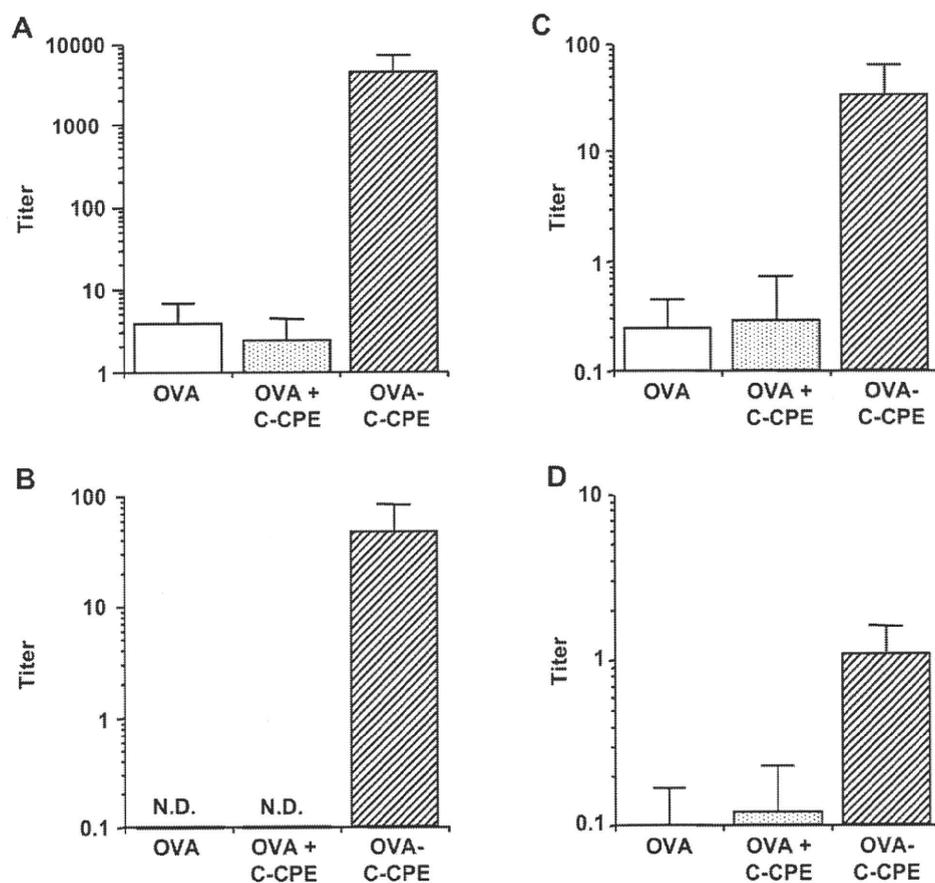


Fig. 3. Production of OVA-specific IgG and IgA by OVA-C-CPE. Mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, or OVA-C-CPE (5 μ g OVA) once a week for 3 weeks. Seven days after the last immunization, the levels of serum IgG (A), nasal IgA (B), vaginal IgA (C) and fecal IgA (D)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

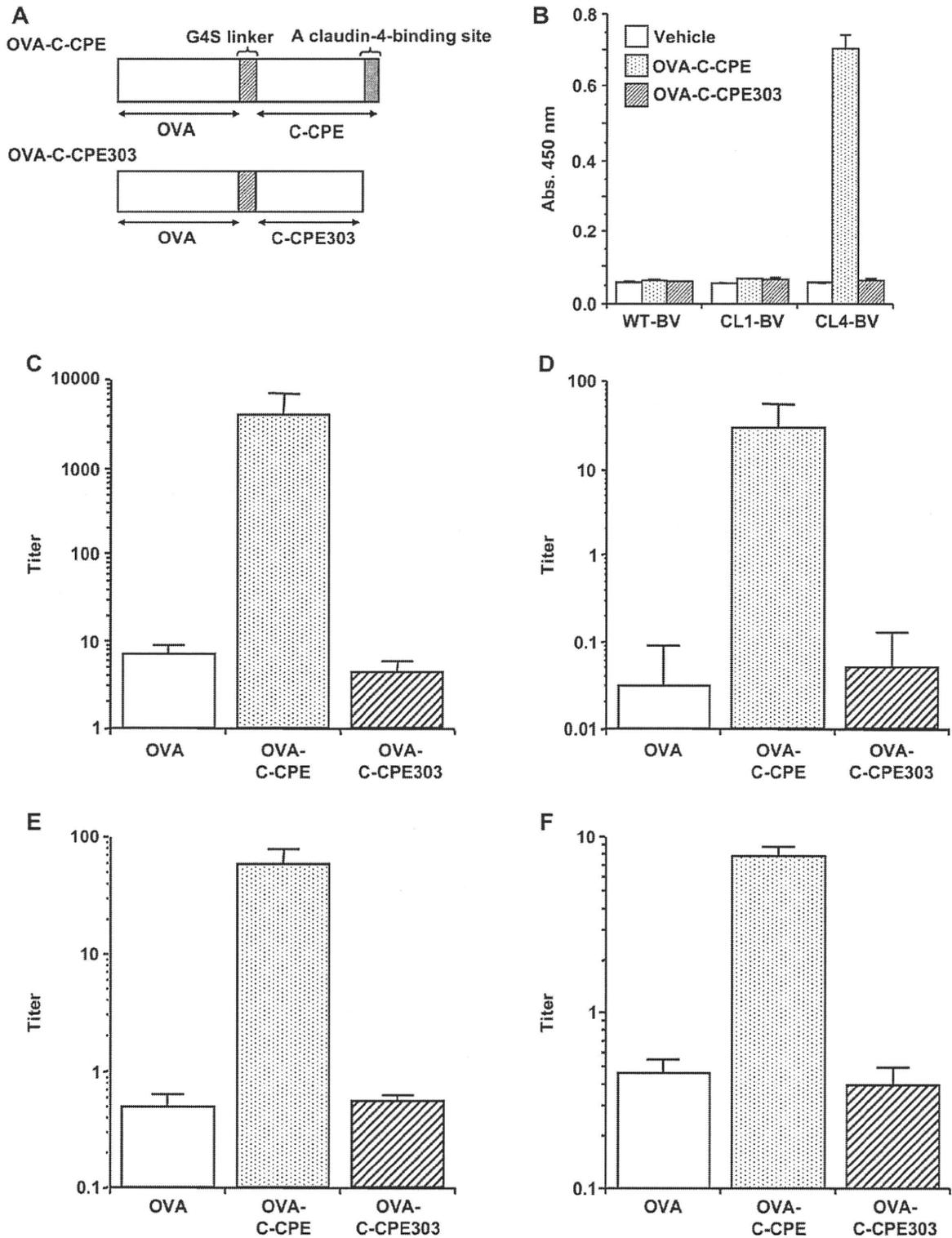


Fig. 4. Involvement of claudin-4 in the immune responses to OVA-C-CPE. **A)** Schematic illustration of OVA-C-CPE mutant. The C-terminal 16 amino acid-deleted C-CPE mutant (C-CPE303) did not bind to claudin-4 [29]. To clarify the involvement of claudin-4 in the immune response initiated by OVA-C-CPE, 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 μ g 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 \pm SD ($n = 4$). Data are representative of three independent experiments.

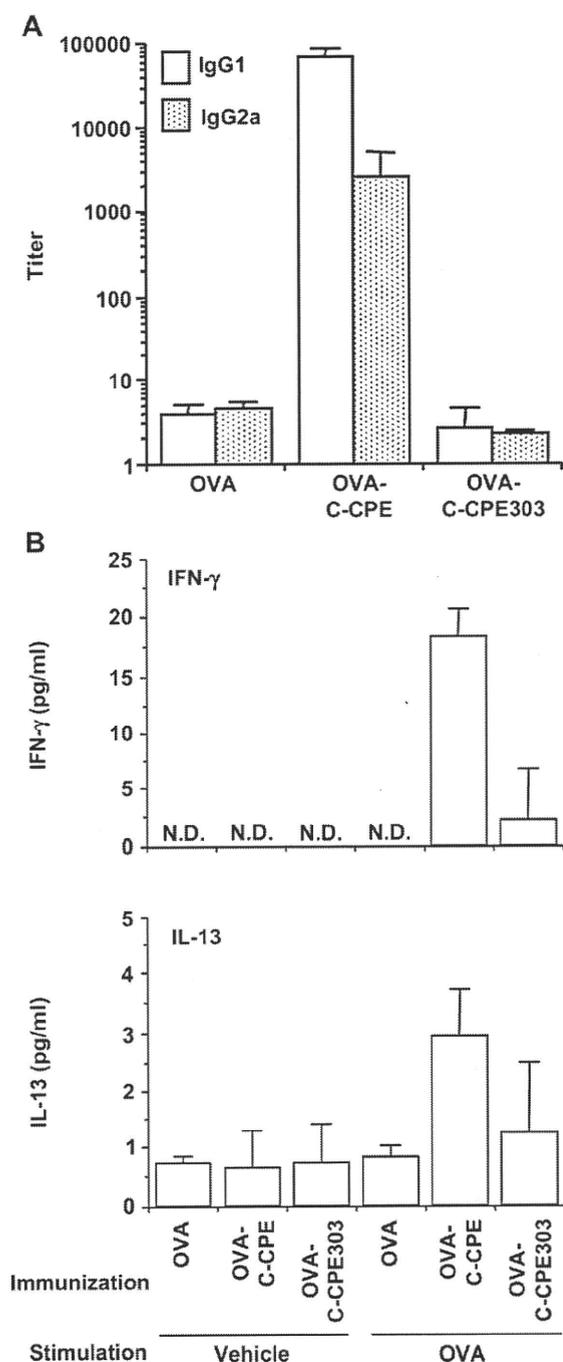


Fig. 5. Th1 and Th2 responses induced by OVA-C-CPE. Mice were nasally immunized with OVA, OVA-C-CPE or OVA-C-CPE303 (5 μ g of OVA) once a week for 3 weeks. Seven days after the last immunization, serum and splenocytes were collected. Serum IgG subclass (IgG1 and IgG2a) was determined by ELISA (A). The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

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

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

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

4. Discussion

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

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

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

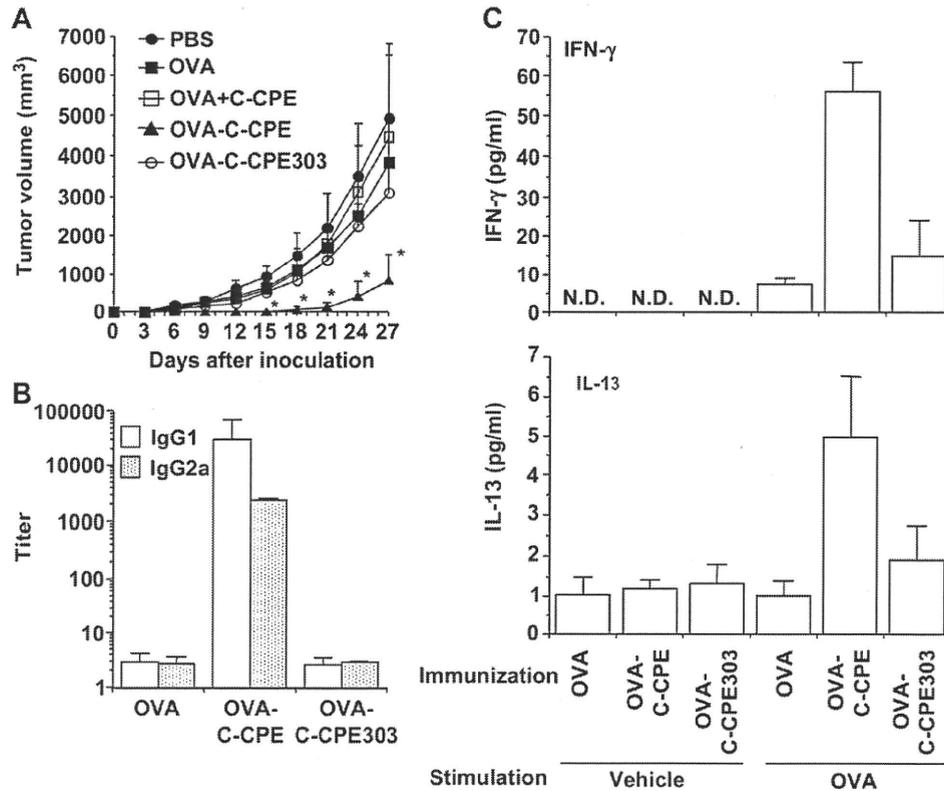


Fig. 6. Anti-tumor activity induced by immunization with OVA-C-CPE in an EG7 cancer model. A) Protective immune response against tumor challenge. C57BL/6 mice were nasally immunized with vehicle, OVA, a mixture of OVA and C-CPE, OVA-C-CPE, or OVA-C-CPE303 (5 μg of OVA) once a week for 3 weeks. Seven 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 \pm SD ($n = 4$). The results are representative of two independent experiments. *Significantly different from the vehicle-immunized group ($P < 0.05$). B, C) Immune responses in the cancer model. Mice were nasally immunized with vehicle, OVA, OVA-C-CPE, or OVA-C-CPE303 (5 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 \pm SD ($n = 4$). N.D., not detected. The results are representative of two independent experiments.

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

Safety is essential for clinical application of the claudin-4-targeting vaccine. Histological injury was not detected after the administration of OVA-C-CPE (data not shown). C-CPE is the receptor-binding domain of CPE without the cytotoxic domain [24,49]. Claudin functions as an epithelial barrier between the

inside and the outside of the body, and modulation of the claudin-4 barrier by the claudin-4-binder may cause side effects due to the non-specific influx of xenobiotics through the loosened epithelial barrier. The claudin family contains more than 20 members, and the claudin expression and barrier-function differ among tissues [38,50]. Expression profiles of claudin in the mucosal epithelium also differ among the sites of epithelium [51,52]. To reduce the risk of solute influx, further investigation of the difference in claudin expression between MALT and the other sites is important. Preparation of a claudin binder with less modulation of the epithelial barrier is also needed.

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

5. Conclusions

In the present study, we prepared C-CPE-fused OVA, and we found that the intranasal administration of the fusion protein increased not only nasal IgA levels but also OVA-specific serum IgG, vaginal IgA and

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

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Hepatoprotective Effect of Syringic Acid and Vanillic Acid on CCl₄-Induced Liver Injury

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The mycelia of the edible mushroom *Lentinula edodes* can be cultured in solid medium containing lignin, and the hot-water extracts (L.E.M.) is commercially available as a nutritional supplement. During the cultivation, phenolic compounds, such as syringic acid and vanillic acid, were produced by lignin-degrading peroxidase secreted from *L. edodes* mycelia. Since these compounds have radical scavenging activity, we examined their protective effect on oxidative stress in mice with CCl₄-induced liver injury. We examined the hepatoprotective effect of syringic acid and vanillic acid on CCl₄-induced chronic liver injury in mice. The injection of CCl₄ into the peritoneal cavity caused an increase in the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. The intravenous administration of syringic acid and vanillic acid significantly decreased the levels of the transaminases. Four weeks of CCl₄ treatment caused a sufficiently excessive deposition of collagen fibrils. An examination of Azan-stained liver sections revealed that syringic acid and vanillic acid obviously suppressed collagen accumulation and significantly decreased the hepatic hydroxyproline content, which is the quantitative marker of fibrosis. Both of these compounds inhibited the activation of cultured hepatic stellate cells, which play a central role in liver fibrogenesis, and maintained hepatocyte viability. These data suggest that the administration of syringic acid and vanillic acid could suppress hepatic fibrosis in chronic liver injury.

Key words hepatoprotection; *Lentinula edodes*; syringic acid; vanillic acid; polyphenol

The edible mushroom *Lentinula edodes* (shiitake) contains bioactive compounds that have immune-modulating, antitumor, antibacterial, antiviral, and antiparasitic effects.^{1–4)} The mycelia of *L. edodes* can be cultured in solid medium, and the hot-water extract (L.E.M.) is commercially available as a nutritional supplement. The main components of L.E.M. are sugars, proteins, and polyphenolic compounds. Polyphenols have protective effects against cancers, cardiovascular disease, and neurodegenerative disorders.^{5–7)} Among polyphenols, syringic acid and vanillic acid are enriched in the solid medium of cultured *L. edodes* mycelia.⁸⁾ *L. edodes* grown in lignocellulose secretes lignin-degrading peroxidase into the culture medium.⁹⁾ The mycelia-derived enzymes degrade the lignin to produce phenolic compounds, particularly syringic acid and vanillic acid. In our previous study, we demonstrated that these phenolic compounds had a hepatoprotective effect on concanavalin A (ConA)-induced liver injury in mice.⁸⁾ We intraperitoneally injected syringic acid or vanillic acid into mice shortly before a ConA injection into the tail vein, which greatly increased the levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In addition, the inflammatory cytokines tumor necrosis factor (TNF)- α , interferon- γ (IFN- γ), and interleukin (IL)-6 in the serum increased rapidly, within 3 h of the ConA administration. The administration of syringic acid or vanillic acid significantly decreased the transaminase and inflammatory cytokine levels and suppressed the disorganization of the hepatic sinusoids. Since ConA-induced liver injury is a mouse model of immune-mediated liver injury that resembles viral and autoimmune hepatitis in humans, the phenolics appeared to have immunomodulating activity.

Polyphenols act as antioxidants by scavenging reactive oxygen species (ROS), which produce oxidative stress and can adversely affect many cellular processes. In the present

study, we examined the possible hepatoprotective effects of two phenolic compounds, syringic acid and vanillic acid, on oxidative stress in chronic CCl₄-induced liver injury in mice. We found that both phenolic compounds could suppress oxidative damage, especially liver fibrosis caused by repeated administration of CCl₄.

MATERIALS AND METHODS

Reagents Syringic acid, vanillic acid, and CCl₄ were purchased from WAKO Pure Chemicals, Co., Ltd. (Osaka, Japan). The chemical structures of syringic acid and vanillic acid were shown in Fig. 1. L.E.M. was obtained from Kobayashi Pharmaceutical Co., Ltd. (Osaka, Japan). CCl₄ was dissolved in olive oil, and L.E.M., syringic acid, and vanillic acid were dissolved in phosphate buffered saline (PBS) for administration into mice. L.E.M., syringic acid, and vanillic acid were dissolved in culture medium for hepatocytes or hepatic stellate cells for *in vitro* experiments.

Animals BALB/c mice and Sprague-Dawley rats were purchased from SLC (Shizuoka, Japan). The animals were housed in an air-conditioned room at 22 °C before the experiment. The animal experiments were conducted according to the ethical guidelines of Osaka University Graduate School

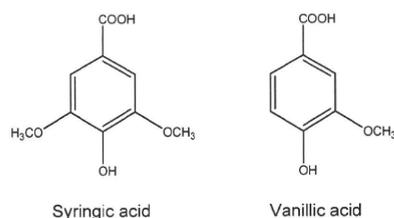


Fig. 1. Chemical Structures of Syringic Acid and Vanillic Acid

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of Pharmaceutical Sciences. The experimental protocol was submitted to the Committee on the Guidelines for Animal Experiments in Graduate School of Pharmaceutical Sciences, and the experiments were conducted after gaining the approval. Mice in the chronic liver injury model received intraperitoneal injections of CCl₄ (0.5 ml/kg body weight) and intravenously administered L.E.M., syringic acid, or vanillic acid (10 mg/kg body weight) twice a week for 4 weeks. Twenty-four hours after the L.E.M., syringic acid, or vanillic acid injection, the mice were anesthetized. Then, blood samples were collected to determine the transaminase activity, and the livers were excised for Azan staining and determination of hydroxyproline and malondialdehyde.

Assays Serum AST and ALT levels were measured by using an assay kit (Transaminase C-II, WAKO, Osaka, Japan).

Histological Analysis Liver specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut from the tissue blocks and mounted on slides. Azan staining was then performed to evaluate the extent of liver fibrosis.

Measurement of Hydroxyproline Content Hepatic hydroxyproline content was measured by using Kivirikko's method¹⁰ with some modifications. Briefly, liver tissue (50 mg) was hydrolyzed with 6 mol/l HCl at 110 °C for 24 h in a glass test tube. After centrifugation at 3000 rpm for 10 min, 2 ml of the supernatant was neutralized with 8 N KOH. Two grams of KCl and 1 ml of 0.5 mol/l borate buffer were then added to the neutralized supernatant, followed by a 15-min incubation at room temperature and then a 15-min incubation at 0 °C. Freshly prepared chloramine-T solution was then added, and the sample was incubated at 0 °C for 1 h, followed by the addition of 2 ml of 3.6 mol/l sodium thiosulfate. The samples were incubated at 120 °C for 30 min. Then, 3 ml of toluene was added, and the samples were incubated for 20 min at room temperature. After centrifugation at 2000 rpm for 5 min, 2 ml of the supernatant was added to 0.8 ml buffer containing Ehrlich's reagent and incubated for 30 min at room temperature. The samples were then transferred to a plastic tube, and the absorbance was measured at 560 nm. The hydroxyproline content was expressed as micrograms of hydroxyproline per gram of liver.

Measurement of Malondialdehyde Lyophilized liver tissue (25 mg) was boiled for 30 min in a solution containing 250 ml of 1.15% KCl, 150 ml of 1% H₃PO₄, and 500 ml of 0.67% thiobarbituric acid. Two milliliters of *n*-butanol was added to the ice-chilled sample, and then the sample was stirred for 30 min. After centrifugation at 3000×g for 10 min, the upper *n*-butanol phase was collected, and the amount of malondialdehyde was colorimetrically determined at 535 and 520 nm.

Isolation and Culture of Hepatic Stellate Cells Hepatic stellate cells (HSCs) were isolated from 10-week-old male Sprague-Dawley rats by digesting the liver with Pronase-E (Merck Darmstadt, Germany) and collagenase type I (WAKO Pure Chemicals Co., Osaka, Japan) as previously described.¹¹ Isolated HSCs were seeded at a density of 2×10⁵ cells/cm² onto 24-well polystyrene culture plates (Asahi Techno Glass, Funabashi, Chiba, Japan) to observe the morphology and analyze fibrosis-related gene expression. Cells were cultured in Dulbecco's modified Eagle's medium

(Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum.

Isolation and Culture of Hepatocytes Hepatocytes were isolated from male BALB/c mice by perfusing the liver with collagenase, according to the method of Seglen.¹² Cells were seeded at a density of 1×10⁵ cells/cm² into multi-well culture plates pre-coated with collagen type I (Asahi Techno Glass, Funabashi, Chiba, Japan). The basal medium consisted of 50 U/ml penicillin G, 50 μg/ml streptomycin (ICN Biochemicals, Inc., Costa Mesa, CA, U.S.A.), 1 μM insulin, 1 μM dexamethasone (WAKO Pure Chemicals Co., Osaka, Japan), and 10% fetal bovine serum in William's medium E (MP Biomedicals, Inc., Kayserberg, France). Six hours after the cells were seeded, the basal medium was replaced with medium containing L.E.M., syringic acid, or vanillic acid at a final concentration of 1.0 mg/ml without insulin and dexamethasone. Cells were then cultured for 24–48 h, and viable cells were counted after trypan blue staining.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) The HSCs were cultured for 7 d and the total RNA was extracted using High Pure RNA Isolation Kit (Roche, Mannheim, Germany). The gene expression of collagen 1 α(I) was analyzed using the following primers: forward 5'-TGCCGTGACCTCAAGATGTG-3' and reverse 5'-CAC-AAGCGTGCTGTAGGTGA-3'. The gene expression of a smooth muscle actin (α-SMA) was analyzed using the following primers: forward 5'-CCGAGATCTCACCGAC-TACC-3' and reverse 5'-TCCAGAGCGACATAGCACAG-3'. The gene expression of β-actin was analyzed using the following primers: forward 5'-CCCAGAGCAAGAGAGGC-ATC-3' and reverse 5'-CTCAGGAGGAGCAATGATCT-3'.

The RT-PCR was examined using RNA PDR Kit (TaKaRa, Kyoto, Japan).

Statistical Analysis The data were analyzed for statistical significance by using Student's *t*-test and Dunnett's test.

RESULTS

Effect on CCl₄-Induced Chronic Liver Injury We examined the hepatoprotective effect of syringic acid and vanillic acid on CCl₄-induced chronic liver injury in mice. As shown in Fig. 2, after 4 weeks of CCl₄ treatment, the activities of blood AST and ALT increased 30-fold and 127-fold, respectively, compared with controls. The intravenous administration of syringic acid or vanillic acid significantly decreased the activities of AST and ALT. These results suggest that syringic acid and vanillic acid suppress the hepatic inflammation caused by repeated CCl₄ treatments. We also examined the effect of syringic acid and vanillic acid on liver fibrogenesis. Figure 3 shows typical Azan staining results, in which fibrous materials are stained blue. In the controls (Fig. 3A), hardly any blue staining was observed in the pericentral area. In contrast, the livers injured by chronic CCl₄ treatment displayed a considerable accumulation of fibrous materials (Fig. 3B). CCl₄ treatment for 4 weeks caused an excessive deposition of collagen fibrils that was sufficient for the evaluation of the antifibrogenic effect of syringic acid and vanillic acid. Based on the results of Azan staining, the syringic acid and vanillic acid treatments obviously suppressed collagen accumulation (Figs. 3D,E). To quantitatively evaluate the effect of syringic acid and vanillic acid on fibrogenesis, we

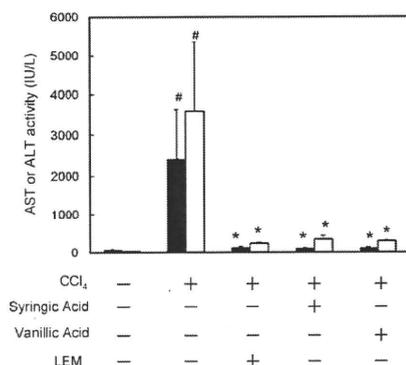


Fig. 2. Effect of Syringic Acid and Vanillic Acid on CCl₄-Induced Chronic Hepatic Injury

Mice received an intraperitoneal injection of CCl₄ and an intravenous injection of L.E.M., syringic acid, or vanillic acid twice a week for 4 weeks. The serum levels of AST (solid column) and ALT (open column) were determined. The values are mean ± S.D. (n=4). The data were analyzed by Student's *t*-test (**p*<0.05, as compared to uninjured control mice) and Dunnett's method (**p*<0.05, as compared to CCl₄-injured control mice).

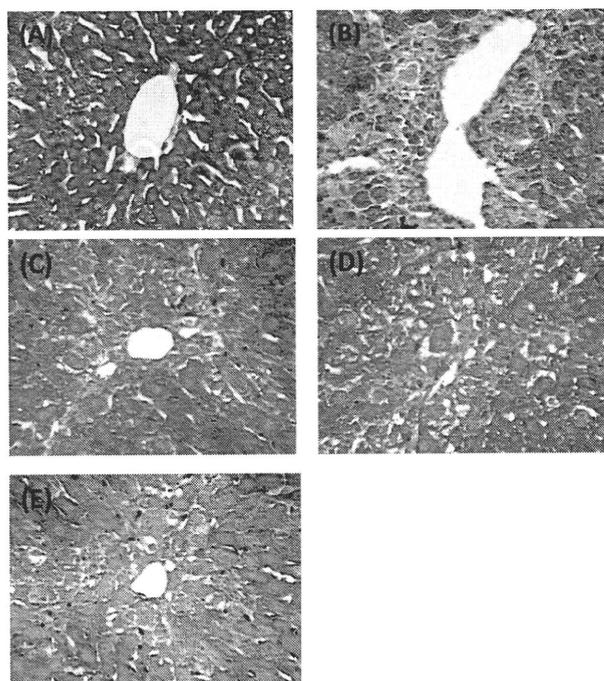


Fig. 3. Azan Staining of Liver Sections

Livers were excised from normal mice (A), CCl₄-injured control mice (B), L.E.M.-treated mice (C), syringic acid-treated mice (D), and vanillic acid-treated mice (E). Original magnification ×400.

measured the hepatic hydroxyproline content, which parallels the extent of fibrosis. After 4 weeks of CCl₄ treatment, the hepatic hydroxyproline content increased 4.6-fold as compared with the controls (Fig. 4). The intravenous administration of syringic acid or vanillic acid significantly decreased the hepatic hydroxyproline content. These data suggest that syringic acid and vanillic acid can suppress hepatic fibrosis in chronic liver injury. Next, we measured the amount of malondialdehyde in the liver samples as a marker of oxidative stress. The malondialdehyde content was drastically increased after 4 weeks of CCl₄ treatment, but the intravenous administration of syringic acid or vanillic acid significantly

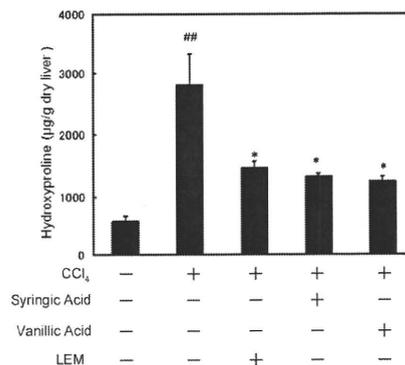


Fig. 4. Effect of Syringic Acid and Vanillic Acid on the Hydroxyproline Content of the Liver

The hydroxyproline content of the liver was measured after 4 weeks of treatments. The values are mean ± S.D. (n=4). The data were analyzed by Student's *t*-test (##*p*<0.01, as compared to uninjured control mice) and Dunnett's method (**p*<0.05, as compared to CCl₄-injured control mice).

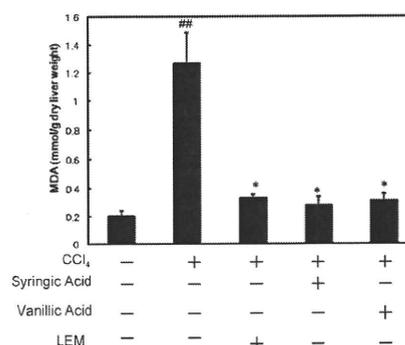


Fig. 5. Effect of Syringic Acid and Vanillic Acid on the Suppression of Oxidative Stress

The malondialdehyde content of the liver was determined after 4 weeks of experiments. The values are mean ± S.D. (n=4). The data were analyzed by Student's *t*-test (##*p*<0.01, as compared to uninjured control mice) and Dunnett's method (**p*<0.05, as compared to CCl₄-injured control mice).

decreased the malondialdehyde content to an almost normal level (Fig. 5). The protective effects of syringic acid and vanillic acid were almost comparable to that of L.E.M. (Figs. 2—5).

In Vitro Effect on HSC Activation and Hepatocyte Viability We examined the direct effect of syringic acid and vanillic acid on the activation of HSCs, which play a central role in liver fibrogenesis, using the monolayer culture. HSCs are activated during the monolayer culture to transform into proliferating myfibroblast-like cells. As shown in Fig. 6A, HSCs were activated after 7 d of culture to be fibroblastic cell-type. The addition of syringic acid or vanillic acid dose-dependently suppressed the activation (Fig. 6B). HSCs maintained their quiescent state by the addition of more than 0.5 mg/ml of the respective compound. Next, the effect of syringic acid and vanillic acid on gene expression of Type I collagen and α-SMA, which are markers of activated HSCs, was examined. HSCs were cultured for 7 d in the presence or absence of syringic acid or vanillic acid, and the gene expression was analysed by RT-PCR. As shown in Fig. 7, syringic acid and vanillic acid remarkably suppressed the expression of collagen and α-SMA genes, indicating that the phenolic compounds directly act on HSCs and suppress the activation

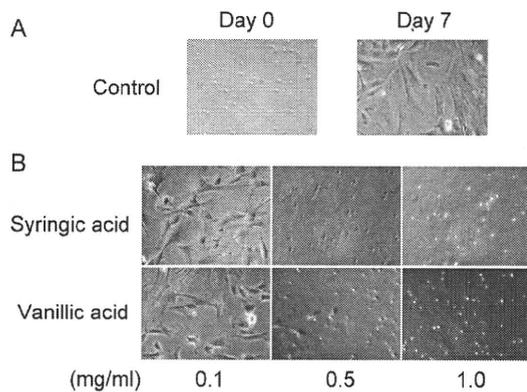


Fig. 6. Phase-Contrast Micrographs of Cultured HSCs

Freshly isolated HSCs were cultured for 7 d in the absence (A) and presence (B) of syringic acid or vanillic acid at the indicated concentration. Original magnification $\times 200$.

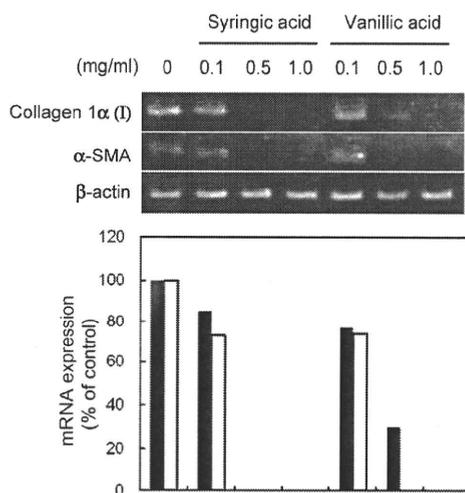


Fig. 7. RT-PCR Analysis of Gene Expression Relating to HSC Activation

Bottom figure shows the relative expression of collagen 1 α (I) (closed bar) and α -SMA (open bar) compared with the non-addition control.

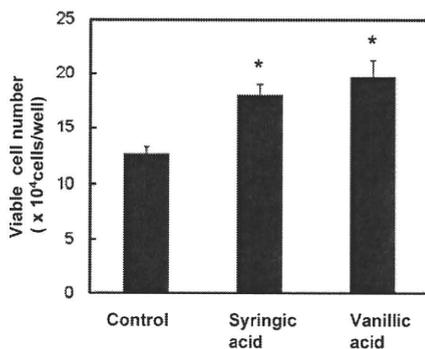


Fig. 8. Effect of Syringic Acid and Vanillic Acid on Viability of Cultured Hepatocytes

Freshly isolated hepatocytes were cultured for 24 h in the absence or presence of 1.0 mg/ml syringic acid or vanillic acid. Viability was measured by trypan blue exclusion test. The data were analyzed by Dunnett's method ($*p < 0.05$, as compared to control).

to maintain the quiescent state. We then examined the effect of the compounds on liver parenchymal hepatocytes using the primary culture (Fig. 8). Hepatocytes were isolated and cultured in the presence or absence of syringic acid or vanil-

lic acid. After 24 h of culture, viable cells were counted using trypan blue exclusion test. The addition of syringic acid or vanillic acid significantly maintained viability of cultured hepatocytes. These results suggested that syringic acid or vanillic acid might suppress liver fibrogenesis and inflammation by inhibiting HSC activation and protecting hepatocytes, respectively in chronically liver injured mice.

DISCUSSION

The physiological functions of plant-derived phenolic compounds have been extensively reported.^{7,13,14} Syringic acid and vanillic acid possess antimicrobial, anti-cancer, and anti-DNA oxidation properties.^{15–17} We recently found that syringic acid and vanillic acid could act as immunomodulators in mice with ConA-induced liver injury.⁸ In the present study, we show that syringic acid and vanillic acid have protective effects in mice with CCl₄-induced liver injury. Both phenolic compounds dramatically suppressed liver fibrogenesis in the chronic CCl₄-treatment model. When these phenolics are orally administered to hamsters, they are adsorbed and appear in the blood within 40 min.¹⁸ Although these compounds are intravenously administered in the present study, oral administration could also elicit the hepatoprotective effect. The syringic acid and vanillic acid contents in L.E.M. are 450 and 378 μ g/g, respectively. Thus, the contents are relatively small, but these compounds are commercially available at low prices. Therefore, syringic acid and vanillic acid might be promising oral agents for the prevention of liver disease.

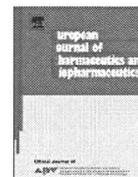
We evaluated the hepatoprotective effect of phenolic compounds in mice with CCl₄-induced liver injury. After intravenous administration, CCl₄ is introduced into the liver, where it is toxic to hepatocytes. Cytochrome P-450 in the endoplasmic reticulum of hepatocytes catalyzes the dehalogenation to produce an unstable complex trichloromethyl radical,¹⁹ resulting in the extensive necrosis of hepatocytes that leads to liver inflammation. In the present study, the transaminase level in the serum was drastically increased by CCl₄ treatment. Generation of ROS degrade polyunsaturated lipids to form malondialdehyde, which is a marker of oxidative stress. The chronic CCl₄ treatment significantly increased the malondialdehyde content of the liver. Syringic acid and vanillic acid clearly suppressed the transaminase and malondialdehyde levels in CCl₄-treated mice. Since both of these compounds have 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity,⁸ the suppression of ROS generation appears to be responsible for the hepatoprotective effect. Moreover, the CCl₄-induced liver fibrogenesis was suppressed by the administration of syringic acid and vanillic acid. The activation of HSCs is responsible for the development of liver fibrosis.^{20,21} During liver injury with persistent inflammation, HSCs are activated to differentiate into proliferating myofibroblast-like cells and overproduce extracellular matrix, leading to fibrogenesis. Since HSCs are activated spontaneously during cell culture,²² we examined the effect of syringic acid and vanillic acid on the activation of primary cell cultures of rat HSCs. Both of these compounds clearly inhibited the change from spherical to spindle shape and the expression of α -smooth muscle actin and collagen Type 1 α genes, which are the markers of HSC activation. We also

examined the effect of syringic acid and vanillic acid on the maintenance of hepatocyte viability *in vitro*. Both of these compounds significantly maintained the viability of primary cell cultures of hepatocytes. Thus, syringic acid and vanillic acid could directly exert a physiological effect on hepatocytes and HSCs. Both phenolic compounds might affect CCl₄ metabolism to inhibit the generation of cytotoxic trichloromethyl radical in the liver. However, the direct effects of syringic acid and vanillic acid on HSCs and hepatocytes were shown in this study, and the protective effect was also shown in ConA-induced liver injured mice in our previous study.⁸⁾ Moreover, these phenolic compounds have strong radical scavenging activity. These results suggest that during the repeated treatment of CCl₄, these compounds could protect hepatocytes and HSCs from CCl₄-induced oxidative stress to suppress liver inflammation and fibrogenesis.

The hot-water extracts from cultured mycelia of *L. edodes* have versatile physiological effects and might contain promising seed compounds for pharmaceutical development. We have shown that syringic acid and vanillic acid have anti-oxidative and immunomodulating activities. In addition to these phenolics, L.E.M. could contain novel compounds with pharmaceutical potential. We are currently trying to isolate bioactive components from L.E.M.

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Research paper

Claudin-4-targeting of diphtheria toxin fragment A using a C-terminal fragment of *Clostridium perfringens* enterotoxinHideki Kakutani^a, Masuo Kondoh^{a,*}, Rie Saeki^a, Makiko Fujii^b, Yoshiteru Watanabe^b, Hiroyuki Mizuguchi^{c,d}, Kiyohito Yagi^{a,*}^a Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan^b Department of Pharmaceutics and Biopharmaceutics, Showa Pharmaceutical University, Tokyo, Japan^c Laboratory of Gene Transfer and Regulation, National Institute of Biomedical Innovation, Osaka, Japan^d Department of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

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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.

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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 recep-

tors 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-disulphophenyl)-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'-GCGGTACCATGGGCGCTGATGATGTTGTTG-3', *KpnI* site is underlined) and a reverse primer (5'-CCTTAATTAATCGCCGTACGCGATTTCCTG-3', *PacI* site is underlined). The resulting PCR fragments were subcloned into *KpnI*/*PacI*-digested pETH₁₀PER (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'-TGGAGGAGGAGGATCTGGAGGAGGAGGATCTGGAGGATACCATACGACGTCCAGACTACGCTAT-3', *PacI* site is underlined) and a reverse oligonucleotide (5'-AGCGTAGTCTGGGACGTCGTATGGGTATCCTCCAGATCCTCCTCCTCAGATCCTCCTCCTCCAAT-3', *PacI* site is underlined). The resulting oligonucleotides were subcloned into *PacI*-digested pET-DTA-C-CPE, and the sequence was confirmed (pET-DTA-linker-C-CPE).

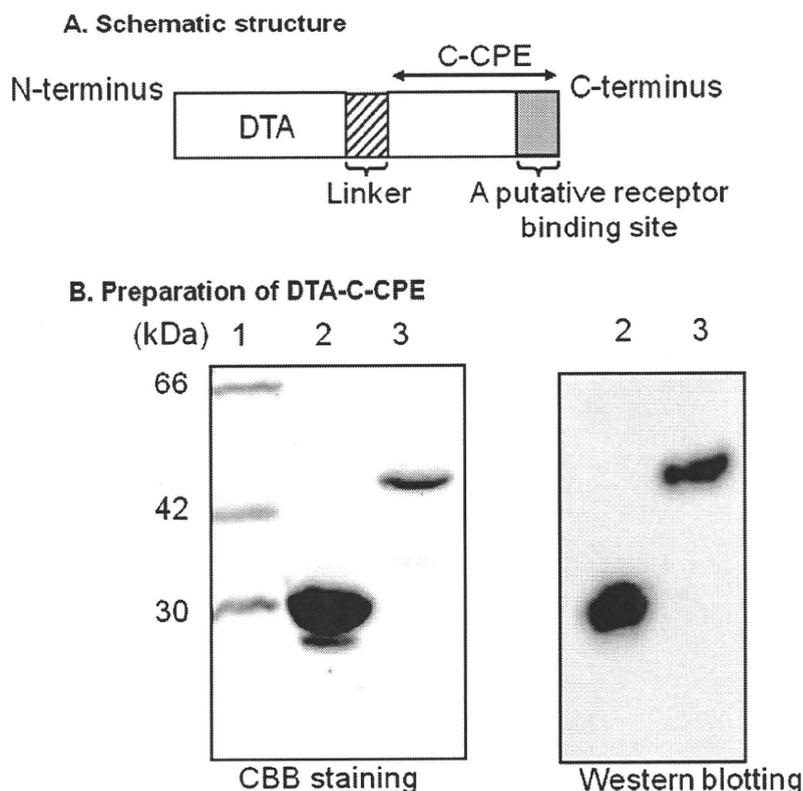


Fig. 1. Preparation of DTA-C-CPE. (A) Schematic structure of DTA-C-CPE. DTA-C-CPE is a fusion protein of DTA and C-CPE with a linker indicated by a slashed column. A dark column indicates a putative receptor-binding region of C-CPE. (B) Preparation of DTA-C-CPE. DTA or DTA-C-CPE was produced by a conventional 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-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- β -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 MgCl₂, 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

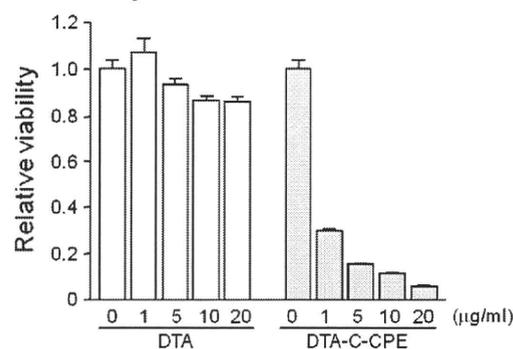
3.1. Preparation of DTA-C-CPE

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

A. WST-8 assay



B. LDH release assay

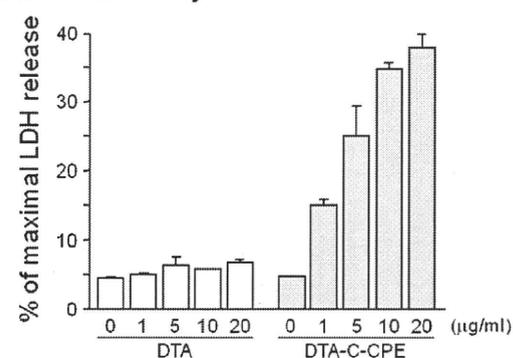


Fig. 2. Cytotoxicity of DTA-C-CPE. CL4/L cells were treated with DTA or DTA-C-CPE at the indicated concentration for 48 h. The cellular viability was measured by WST-8 assay (A) or LDH-release assay (B). Data are the mean \pm SD ($n = 3$). The data are representative of three independent experiments.

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%.

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. DTA-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

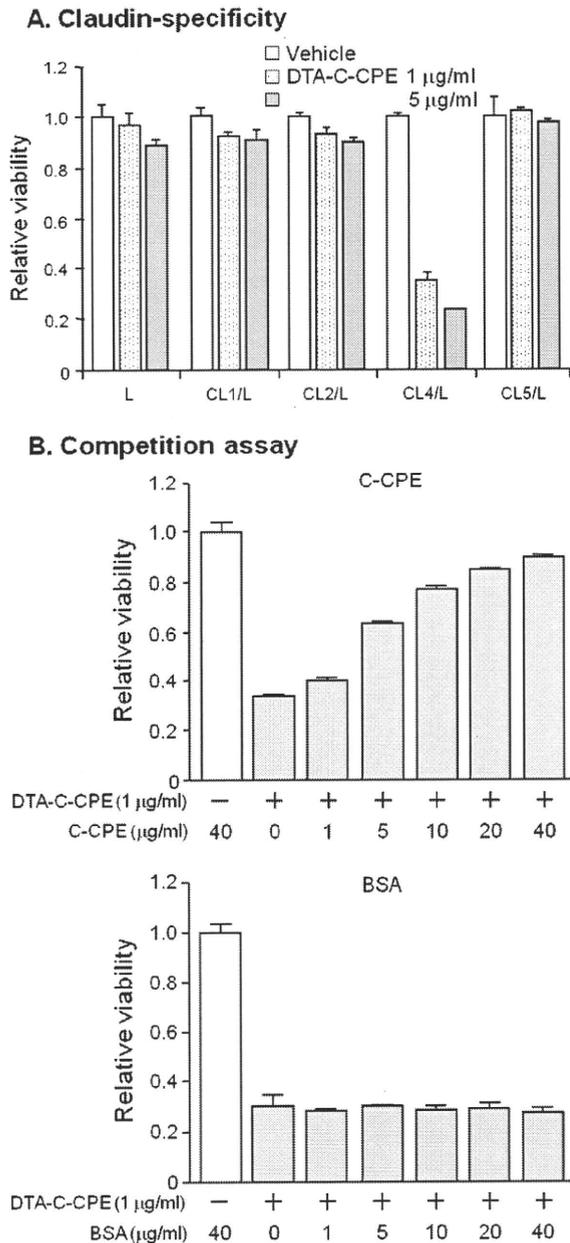


Fig. 3. Cytotoxic properties of DTA-C-CPE. (A) Claudin-specificity. L, CL1/L, CL2/L, CL4/L or CL5/L cells were treated with DTA-C-CPE at the indicated concentration for 48 h. After incubation, the cellular viability was measured by WST-8 assay. Data are the mean \pm SD ($n=3$). The data are representative 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 \pm 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 vesicles, 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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