EFFECTS OF *Clostridium perfringens* ENTEROTOXIN VIA CLAUDIN-4 ON NORMAL HUMAN PANCREATIC DUCT EPITHELIAL CELLS AND CANCER CELLS

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Abstract: The tight junction protein claudin-4 is frequently overexpressed in pancreatic cancer, and is also a receptor for *Clostridium perfringens* enterotoxin (CPE). The cytotoxic effects of CPE are thought to be useful as a novel therapeutic tool for pancreatic cancer. However, the responses to CPE via claudin-4 remain unknown in normal human pancreatic duct epithelial (HPDE) cells. We introduced the human telomerase reverse transcriptase (hTERT) gene into HPDE cells in primary culture as a model of normal HPDE cells in vitro. hTERT-HPDE cells treated with or without 10% FBS and pancreatic cancer cell lines PANC-1, BXPC3, HPAF-II and HPAC were treated with CPE. In Western blotting, the expression of claudin-4 protein in hTERT-HPDE cells treated with 10% FBS was as high as it was in all of the pancreatic cancer cell lines. In hTERT-HPDE cells with or without 10% FBS, cytotoxicity was not observed at any concentration of CPE, whereas in all pancreatic cancer cell lines, CPE had a dose-dependent cytotoxic effect. In hTERT-HPDE cells with 10% FBS, claudin-4 was localized in the apical-most regions, where there are tight junction areas, in which in all pancreatic cancer cell lines claudin-4 was found not only in the apical-most regions but also at basolateral membranes. In hTERT-HPDE...
cells with 10% FBS after treatment with CPE, downregulation of barrier function and claudin-4 expression at the membranes was observed. In HPAC cells, the sensitivity to CPE was significantly decreased by knockdown of claudin-4 expression using siRNA compared to the control. These findings suggest that, in normal HPDE cells, the lack of toxicity of CPE was probably due to the localization of claudin-4, which is different from that of pancreatic cancer cells. hTERT-HPDE cells in this culture system may be a useful model of normal HPDE cells not only for physiological regulation of claudin-4 expression but also for developing safer and more effective therapeutic methods targeting claudin-4 in pancreatic cancer.

Key words: Tight junction, Claudin-4, CPE, Human pancreatic duct epithelial cells, Human pancreatic cancer cells

INTRODUCTION

In some human cancers, including ovarian, breast, pancreatic and prostate cancers, tight junction protein claudins are abnormally regulated and are thus promising molecular targets for diagnosis and therapy [1, 2]. The claudin family, which consists of at least 24 members, is solely responsible for forming tight junction strands and has four transmembrane domains and two extracellular loops [3]. The second extracellular loop is the receptor of Clostridium perfringens enterotoxin (CPE)[4]. This 35-kDa polypeptide causes food poisoning in humans. CPE binds to its receptor, and then CPE causes changes in the membrane permeability via complex formation on the plasma membrane followed by the induction of apoptosis [5]. In pancreatic cancer, claudin-4 is frequently overexpressed [6] and is a high-affinity receptor of CPE [7]. It is anticipated that it may be possible to develop a novel tumor-targeted therapy for pancreatic cancer using a claudin-4-targeting molecule.

Although better knowledge of the regulation of claudin-4 and the responses to cytotoxic CPE via claudin-4 in normal human pancreatic duct epithelial (HPDE) cells is essential to develop safer and more effective diagnostic and therapeutic methods targeting this protein in pancreatic cancer, these mechanisms remain unknown partly because primary cultures of normal HPDE cells are difficult to convert into continuously growing cultures.

Thus, we previously introduced the hTERT gene into HPDE cells in primary culture as a model of normal HPDE cells [8, 9]. The properties of hTERT-HPDE cells, including HPDE cell markers CK7, CK19 and carbonic anhydrase isozyme 2 (CA-II), as well as epithelial tight junction molecules claudin-1, -4, -7 and -18, occludin, JAM-A, ZO-1, ZO-2 and tricellulin, are similar to those of HPDE cells in vivo [8, 9]. In hTERT-HPDE cells, the tight junction molecules, including claudin-4, and the barrier function are upregulated by 10% FBS [8].

In the present study, to investigate the effects of CPE on normal HPDE cells, we compared hTERT-HPDE cells highly expressing claudin-4 treated with 10% FBS and human pancreatic cancer cell lines.
MATERIALS AND METHODS

Isolation and culture of human pancreatic duct epithelial (HPDE) cells

Human pancreatic tissues were obtained from patients with pancreatic or biliary tract diseases who underwent pancreatic resection in the Sapporo Medical University hospital. Informed consent was obtained from all patients, and the study was approved by the ethics committee of Sapporo Medical University. The procedures for primary culture of human pancreatic duct epithelial cells (HPDE cells) were as reported previously [8, 9]. Some primary cultured HPDE cells were transfected with the catalytic component of telomerase, the human catalytic subunit of the telomerase reverse transcriptase (hTERT) gene as described previously [8, 9]. The hTERT-HPDE cells were plated on 35-mm and 60-mm culture dishes or 24-well tissue culture plates (Corning Glass Works, Corning, NY, USA), which were coated with rat tail collagen (500 µg of dried tendon/ml 0.1% acetic acid). The cells were cultured in serum-free bronchial epithelial cell basal medium (BEBM, Lonza Walkersville, Inc.; Walkersville, MD, USA) supplemented with bovine pituitary extract (1% v/v), 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 50 µg/ml gentamicin, 50 µg/ml amphotericin B, 0.1 ng/ml retinoic acid, 10 µg/ml transferrin, 6.5 µg/ml triiodothyronine, 0.5 µg/ml epinephrine, 0.5 ng/ml epidermal growth factor (Lonza Walkersville, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) and incubated in a humidified, 5% CO2:95% air incubator at 37°C. In this experiment, 2nd and 3rd passaged cells were used. Some cells were treated with 10% FBS for 24 h.

Culture of pancreatic cancer cell lines

Human pancreatic cancer cell lines PANC-1, HPAF-II, BXPC-3 and HPAC were purchased from ATCC (Manassas, VA). PANC-1 cells and HPAC cells were maintained with DMEM (Sigma-Aldrich) supplemented with 10% dialyzed fetal bovine serum (FBS, Invitrogen; Carlsbad, CA). HPAF-II cells were maintained with DMEM containing 10% FBS, and supplemented with 0.1 mM non-essential amino acids (Sigma-Aldrich) and 1 mM sodium pyruvate (Sigma-Aldrich). BXPC-3 was maintained with RPMI-1640 (Sigma-Aldrich) supplemented with 10% FBS. The medium for all cell lines contained 100 U/ml penicillin and 100 µg/ml streptomycin and all cells were plated on 35- and 60-mm culture dishes (Corning Glass Works, Corning, NY) that were coated with rat tail collagen (500 µg of dried tendon/ml in 0.1% acetic acid), and incubated in a humidified 5% CO2 incubator at 37°C.

For RNA interference studies, small interference RNA (siRNA) duplexes targeting the mRNA sequences of human claudin-4 were purchased from Invitrogen (Carlsbad, CA). The sequences were as follows: siRNA-1 of claudin-4 (sense 5’- GCAACAUUGUACCUCGACGACAU-3’, antisense 5’- AUGGUCUGCGAGGUGACAAUGUUGC-3’); and siRNA-2 of claudin-4 (sense 5’- UCCUGUUGGCGCCCUUAUGGUGAU-3’, antisense 5’- AUCACCAUAAGGCGCGCCAACAGGA-3’). At one day before
transfection, the HPAC cells were plated in medium without antibiotics such that they would be half confluent at the time of transfection. The cells were transfected with 100 nM siRNAs using Lipofectamine RNAiMAX (Invitrogen) as a carrier according to the manufacturer’s instructions.

**Western blot analysis**

For Western blotting of total cell lysates, the dishes were washed with PBS and 300 µl of sample buffer (1 mM NaHCO₃ and 2 mM phenylmethylsulfonyl fluoride) was added to 60-mm culture dishes. The cells were scraped and collected in microcentrifuge tubes and then sonicated for 10 s. The protein concentrations of samples were determined using a BCA Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL). Aliquots of 15 µg of protein/lane for each sample were separated by electrophoresis in 4/20% SDS polyacrylamide gels (Cosmo Bio Co., Tokyo, Japan). After electrophoretic transfer to nitrocellulose membranes (Immobilon; Millipore, Billerica, MA), the membranes were saturated with blocking buffer (Tris-buffered saline [TBS] with 0.1% Tween 20 and 4% skim milk) for 30 min at room temperature and incubated with polyclonal anti-claudin-1, anti-claudin-4, anti-claudin-7, anti-occludin (Zymed Laboratories, San Francisco, CA), and polyclonal anti-actin (Sigma-Aldrich) antibodies for 1 h at room temperature. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Dako A/S, Copenhagen, Denmark) at room temperature for 1 h. The immunoreactive bands were detected using an ECL Western blotting analysis system (GE Healthcare, Little Chalfont, UK).

**Immunostaining**

The cells were grown on 35-mm glass base dishes (Iwaki, Chiba, Japan) coated with rat tail collagen and incubated with 10% FBS. The cells were fixed with cold acetone and ethanol (1:1) at -20°C for 10 min. After rinsing in PBS, the sections and the cells were incubated with a monoclonal claudin-4 antibody (3E2C1, Zymed Laboratories, San Francisco, CA) at room temperature (RT) for 1 h and then with Alexa 594 (red)-conjugated anti-mouse IgG (Molecular Probes Inc., Eugene, OR) at RT for 1 h. DAPI (Sigma-Aldrich) was used for counterstaining of nuclei in the cells. The specimens were examined using an epifluorescence microscope (Olympus, Tokyo, Japan) and a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany).

**Measurement of transepithelial electrical resistance (TER)**

The cells were cultured to confluence on inner chambers of 12-mm Transwell with 0.4-µm pore size filters (Corning Life Science). TER was measured using an EVOM voltmeter with an ENDOHM-12 (World Precision Instruments, Sarasota, FL) on a heating plate (Fine, Tokyo, Japan) adjusted to 37°C. The values are expressed in standard units of ohms per square centimeter and presented as the mean ± SD of the triplicate experiments. For calculation, the resistance of blank filters was subtracted from that of filters covered with cells.
Measurement of permeability
To determine the paracellular flux, the cells were cultured on 12 mm Transwell, 0.4-μm pore size filters (Coming Inc.), and then FITC-labeled dextran (MW: 7 kDa, Sigma-Aldrich)-containing medium was added to the inner chamber. Samples were collected from the outer chamber at 60 min and were measured with a Wallac 1420 multilabel counter (PerkinElmer, Turku, Finland).

CPE treatment and MTT assay
For purification of CPE, the bacterial DNA fragment encoding the full-length CPE gene was PCR amplified (primer 1, 5'-AGA TGT TAA TCA TAT GAT GCT TAG TAA CAA TTT AAA TCC-3'; primer 2, 5'-AGG ATC CTT AAA ATT TTT GAA ATA ATA TTG AAT AAG GG-3'). The PCR products were digested with the restriction enzymes Ndel/BamHI and cloned into an Ndel/BamHI-digested pet 16 (Novagen) expression vector to generate an in-frame NH2-terminus His-tagged CPE expression plasmid, pet 16-(His)5-CPE. His-tagged CPE toxin was prepared from pet 16-(His)5-CPE transformed Escherichia coli BL-21 (DE 3). Transformed bacteria were grown overnight at 37°C, after which CPE protein expression was induced for 3 h with 1 mM isopropyl β-D-thiogalactoside. The cells were harvested, resuspended in 20 mM Tris-HCl, pH 7.9, binding buffer, and lysed by sonication. The fusion protein was isolated from the supernatant on a His-Bind column (Novagen). After treatment with 10-column volumes of binding buffer and six-column volumes of wash buffer, His-tagged CPE was eluted with 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and dialyzed (Mr 3,500 cutoff dialysis tubing) against PBS overnight. The cells plated on 24-well tissue culture plates (BD Labware, Franklin Lakes, NJ) were treated with 0.1-10 μg/ml CPE for 1 h or 2 h. Cell survival was evaluated with a colorimetric assay using an MTT Cell Growth Assay Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. The ratio of absorbance was calculated and presented as the mean ± SD of triplicate experiments.

Data analysis
Differences between groups were tested by ANOVA followed by a post-hoc test and an unpaired two-tailed Student’s t test and considered to be significant when p < 0.05.

RESULTS
Expression of claudin-4 protein in hTERT-HPDE cells and pancreatic cancer cell lines
We previously reported that in hTERT-HPDE cells, a model of normal human pancreatic duct cells in vitro, claudin-4 protein could be markedly induced by treatment with 10% FBS for 24 h [8]. In the present study, we investigated the
Fig. 1. Characterization of hTERT-HPDE cells and pancreatic cancer cell lines. A – Phase-contrast images of hTERT-HPDE cells with or without 10% FBS and pancreatic cancer cell lines PANC-1, BXPC-3, HPAF-II, and HPAC. Bar: 40 μm. B – Western blotting for claudin-4 in hTERT-HPDE cells with or without 10% FBS and pancreatic cancer cell lines PANC-1, BXPC-3, HPAF-II, and HPAC. Actin is expression control. C – TER values of hTERT-HPDE cells with or without 10% FBS and pancreatic cancer cell lines PANC-1, BXPC-3, HPAF-II, and HPAC. Data represent the mean ± SD (n = 6).
expression of claudin-4 protein in hTERT-HPDE cells with or without 10% FBS compared to pancreatic cancer cell lines PANC-1, BXPC3, HPAF-II and HPAC. All the cells had a small cobblestone appearance in phase-contrast images (Fig. 1A). In Western blotting, the expression of claudin-4 protein in hTERT-HPDE cells with 10% FBS was as high as in all the pancreatic cell lines, whereas it was not detected in hTERT-HPDE cells without 10% FBS (Fig. 1B).

**Barrier function of hTERT-HPDE cells and pancreatic cancer cell lines**

The barrier function of hTERT-HPDE cells with or without 10% FBS, pancreatic cancer cell lines PANC-1, BXPC3, HPAF-II and HPAC was measured by TER values under confluent conditions. The TER values of hTERT-HPDE cells with or without 10% FBS, PANC-1, BXPC3, HPAF-II and HPAC were 6 ± 1, 150 ± 28, 6 ± 2, 7 ± 1, 480 ± 88, and 345 ± 55 ohm cm², respectively (Fig. 1C).

![Graphs showing cell viability and TER values](image-url)

Fig. 2. Cytotoxicity of CPE in hTERT-HPDE cells with or without 10% FBS and pancreatic cancer cell lines PANC-1, BXPC-3, HPAF-II, and HPAC. Cell viability (%) was measured using an MTT assay kit according to the manufacturer’s instructions. Data represent the mean ± SD (n = 6). *p < 0.05 vs control.
Effects of CPE in hTERT-HPDE cells and pancreatic cancer cell lines
To investigate the cytotoxicity of CPE in hTERT-HPDE cells, a model of normal human pancreatic duct cells in vitro, hTERT-HPDE cells with or without 10% FBS were treated with 0.25-4 μg/ml CPE for 1 h and measured using MTT assay. Cytotoxicity was not observed at any concentration of CPE in hTERT-HPDE cells with or without 10% FBS (Fig. 2). When the pancreatic cancer cell lines were treated with 0.25-2 μg/ml CPE for 1 h, CPE led to dose-dependent cytotoxic effects in all the pancreatic cell lines; in PANC-1 and BXPC3 from 0.25 μg/ml CPE, in HPAF-II and in HPAC from 0.5 μg/ml CPE (Fig. 2).

Localization of claudin-4 protein in hTERT-HPDE cells and pancreatic cancer cell lines
To investigate the detailed localization of claudin-4 in hTERT-HPDE cells and pancreatic cancer cell lines, immunocytochemistry was carried out using a confocal laser scanning microscope. In hTERT-HPDE cells with 10% FBS, claudin-4 was localized in the apical-most regions, which were tight junction areas, whereas it was not detected at the membranes in hTERT-HPDE cells without 10% FBS (Fig. 3). In all pancreatic cell lines, claudin-4 was found not only in the apical-most regions but also at basolateral membranes (Fig. 3).

Fig. 3. Immunocytochemistry for claudin-4 in hTERT-HPDE cells with or without 10% FBS and pancreatic cancer cell lines PANC-1, BXPC-3, HPAF-II, and HPAC. Bars: 10 μm.
Effects of CPE on barrier function and claudin-4 expression of hTERT-HPDE cells
To investigate the effects of CPE on barrier function and claudin-4 expression of hTERT-HPDE cells as normal HPDE cells, hTERT-HPDE cells with 10% FBS cultured on Transwell plates were treated with 4 μg/ml CPE for 1 h or 2 h and 10 μg/ml CPE for 1 h into both inner and outer chambers. In MTT assay,

![Graphs showing cell viability, TER values, and paracellular flux using FITC-dextran.](image)

Fig. 4. Cytotoxicity (A), TER values (B) and paracellular flux using FITC-dextran (C) in hTERT-HPDE with 10% FBS treated with 4 μg/ml CPE for 1 h and 2 h and 10 μg/ml CPE for 1 h. Cell viability (%) was measured using an MTT assay kit according to the manufacturer’s instructions. Data represent the mean ± SD (n = 6). *p < 0.05 vs control. D – Immunocytochemistry for claudin-4 in hTERT-HPDE cells with 10% FBS treated with 4 μg/ml CPE for 2 h. Bar: 10 μm.
cytotoxicity was not observed in all cells treated with CPE (Fig. 4A). In barrier function, a significant decrease of TER values and a significant decrease of paracellular flux of FITC-dextran were observed in all cells after treatment with CPE (Fig. 4B, C). In immunocytochemistry, claudin-4 expression showed punctuate distribution and was decreased at the cell membranes at 2 h after treatment with 4 mg/ml CPE (Fig. 4D).

Fig. 5. Western blotting (A) for claudin-1, -4, -7 and occludin, and immunocytochemistry (B) for claudin-4 in HPAC cells treated with siRNAs of claudin-4. Bar: 10 μm.

C - Cytotoxicity of CPE in HPAC cells treated with siRNAs of claudin-4. Cell viability (%) was measured using an MTT assay kit according to the manufacturer’s instructions. Data represent the mean ± SD (n = 6). *p < 0.05 versus control.
Effects of CPE in pancreatic cancer cell line HPAC cells treated with siRNA of claudin-4

To investigate whether claudin-4 expression was directly associated with the toxicity of CPE in HPAC cells, knockdown of claudin-4 was performed using siRNAs. At 48 h after transfection with two sets of 100 nM siRNAs of claudin-4, claudin-4 protein was decreased compared to the control in Western blotting, whereas no changes in expression of occludin and claudin-1 or -7 were observed (Fig. 5). At 48 h after transfection with 100 nM siRNA-1 of claudin-4, claudin-4 protein was decreased and disappeared at the membranes (Fig. 5). The cytotoxicity from 0.5 μg/ml CPE was significantly decreased by two sets of 100 nM siRNAs of claudin-4 compared to the control (Fig. 5).

DISCUSSION

In the present study, by using hTERT-HPDE cells that showed properties similar to those of normal HPDE cells in vivo, we first demonstrated the lack of cytotoxicity of CPE via claudin-4 in normal HPDE cells. This was probably because claudin-4 was localized at only tight junction areas. CPE leads to an acute dose-dependent cytotoxic effect in claudin-4-expressing nude mouse xenografts of the PANC-1 cell line [6]. However, the effects of CPE on pancreatic cancer cells in vitro remain unknown. It is reported that PANC-1 and BXPC3 are poorly differentiated types, and that HPAF-II and HPAC are moderately or well differentiated [10]. In the present study, CPE led to an acute dose-dependent cytotoxic effect in all pancreatic cell lines (PANC-1, BXPC3, HPAF-II and HPAC) in vitro. Furthermore, in HPAC cells, the cytotoxicity of CPE was significantly decreased by knockdown of claudin-4 using siRNAs. These culture systems may play a crucial role in examination of the effects of CPE on various differentiated pancreatic cancer cells in vitro.

It is thought that CPE binds to the free second extracellular loop of claudins outside tight junctions [11]. In the present study, in hTERT-HPDE cells with 10% FBS, claudin-4 was localized in the apical-most regions, which were tight junction areas, whereas it was not detected at the membranes in hTERT-HPDE cells without 10% FBS. In all pancreatic cell lines, claudin-4 was found not only in the apical-most regions but also at basolateral membranes. These findings suggested that in pancreatic cancer cells, CPE also bound to the free second extracellular loop of claudin-4 outside tight junctions and that, in normal HPDE cells, it could not bind to that of claudin-4 in tight junction areas.

It is known that in MDCKI cells treated with C-CPE (COOH-terminal half fragment of CPE), claudin-4 was selectively removed from tight junction areas with its concomitant degradation, and the barrier function and the tight junction strands were decreased [12]. In the present study, to investigate the effects of CPE on barrier function and claudin-4 expression of normal HPDE cells, hTERT-HPDE cells with 10% FBS cultured on Transwell plates were treated with 4 μg/ml CPE for 1 h or 2 h and 10 μg/ml CPE for 1 h. In all cells treated
with CPE in which cytotoxicity was not observed, the barrier functions measured by TER values and paracellular flux of FITC-dextran were significantly decreased compared to the control and claudin-4 expression was also decreased at the cell membranes. These findings suggest that in normal HPDE cells, CPE may affect barrier function and claudin-4 expression at the membranes but not cytotoxicity.

The claudin family consists of at least 24 members but not all of the members serve as receptors for CPE. It was reported that claudin-3, -4, -6, -7, -8, and -14, but not claudin-1, -2, -5, and -10, were sensitive to CPE [4]. In particular, claudin-4, which is overexpressed in pancreatic cancer cells, is a high-affinity receptor of CPE [6, 7]. In hTERT-HPDE cells, mRNAs of claudin-1, -4, -7, -8 and -18 were detected in RT-PCR, and expression of claudin-1, -4, and -7 was induced by treatment with 10% FBS [8]. Since in hTERT-HPDE cells with 10% FBS, cytotoxicity was not observed after treatment with CPE in the present study, in normal HPDE cells, the cytotoxicity of CPE via claudin-7 and -8 may be limited as well as via claudin-4.

Although the expression and localization of claudin-4 were almost the same in all the pancreatic cell lines, the dose level of CPE for cytotoxicity was different; for PANC-1 and BXPC3 it was from 0.25 μg/ml CPE, while for HPAF-II and HPAC it was from 0.5 μg/ml CPE. PANC-1 and BXPC3 had a low level of epithelial barrier function indicated as TER values, whereas HPAF-II and HPAC had higher barrier function than that of hTERT-HPDE cells with 10% FBS. It is possible that since the barrier functions of PANC-1 and BXPC3 cells are at a very low level, CPE can easily reach claudin-4 on the basolateral membrane. These observations suggest that the cytotoxicity of CPE was stronger against poorly differentiated pancreatic cancers than well-differentiated pancreatic cancers, and that it might be closely associated with not only the expression and the localization of claudin-4 but also the barrier function.

In conclusion, the hTERT-HPDE cells in this culture system provide us with an indispensable and stable model of normal HPDE cells not only for physiological regulation of claudin-4 expression but also for developing safer and more effective therapeutic methods targeting claudin-4 in pancreatic cancer.

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