In vitro cytotoxicity induced by Clostridium perfringens isolate carrying a chromosomal cpe gene is exclusively dependent on sporulation and enterotoxin production

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ABSTRACT

Clostridium perfringens type A is a common source of food poisoning (FP) and non-food-borne (NFB) gastrointestinal diseases in humans. In the intestinal tract, the vegetative cells sporulate and produce a major pathogenic factor, C. perfringens enterotoxin (CPE). Most type A FP isolates carry a chromosomal cpe gene, whereas NFB type A isolates typically carry a plasmid-encoded cpe. In vitro, the purified CPE protein binds to a receptor and forms pores, exerting a cytotoxic activity in epithelial cells. However, it remains unclear if CPE is indispensable for C. perfringens cytotoxicity. In this study, we examined the cytotoxicity of cpe-harboring C. perfringens isolates co-cultured with human intestinal epithelial Caco-2 cells. The FP strains showed severe cytotoxicity during sporulation and CPE production, but not during vegetative cell growth. While Caco-2 cells were intact during co-culturing with cpe-null mutant derivative of strain SM101 (a FP strain carrying a chromosomal cpe gene), the wild-type level cytotoxicity was observed with cpe-complemented strain. In contrast, both wild-type and cpe-null mutant derivative of the NFB strain F4969 induced Caco-2 cell death during both vegetative and sporulation growth. Collectively, the Caco-2 cell cytotoxicity caused by C. perfringens strain SM101 is considered to be exclusively dependent on CPE production, whereas some additional toxins should be involved in F4969-mediated in vitro cytotoxicity.

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1. Introduction

Clostridium perfringens, a Gram-positive spore-forming anaerobic bacterium, is a major pathogen of humans and domestic animals [1]. The virulence of C. perfringens is largely attributed to its toxin-producing ability and isolates of this organism are classified into five types (type A to E) based upon the production of four major toxins (α, β, ε, and α) [2]. The type A strains that produce α- but not β-, ε-, or α-toxin, are important cause of histotoxic infections like gas gangrene in humans [3]. Some (less than 5% of global isolates) C. perfringens isolates produce another important toxin named C. perfringens enterotoxin (CPE), which is responsible for food poisoning (FP) and non-food-borne (NFB) gastrointestinal (GI) diseases such as antibiotic-associated diarrhea and sporadic diarrhea [4]. Most type A FP isolates carry a chromosomal cpe gene, whereas NFB type A isolates typically carry a plasmid-borne cpe gene [5]. C. perfringens type A FP consistently ranks among the most common bacterial food-borne illnesses in the USA, UK, and Japan [6–8]. On the other hand, type A strains carrying a plasmid-encoded cpe gene cause ~5–10% of all cases of human NFB GI diseases [5].

In the intestinal tract, C. perfringens vegetative cells sporulate and produce CPE in the mother cell compartment of sporulating cells [9,10]. At the completion of sporulation, the mother cell lyses,
and CPE is released in the intestinal lumen [11,11]. The released CPE then binds to enterocyte receptors, certain members of the claudin-family on the tight junction [12,13]. The bound CPE oligomerizes and perforates the plasma membrane leading to diarrhea and abdominal cramps [14–16]. Several experimental evidences proved that GI symptoms of C. perfringens-associated diseases are caused by the CPE. Human volunteer feeding experiments have demonstrated that ingestion of purified CPE reproduces diarrhea and abdominal cramping [17]. In rabbit or mouse intestinal loop models, purified CPE injection mediates tissue damage and fluid accumulation in the intestine [18–20]. Sarker et al. demonstrated that CPE expression is necessary and sufficient for C. perfringens strains SM101 and F4969 to cause fluid accumulation and GI damage in a rabbit ileal loop model using the lysates of sporulating cultures of cpe-knock out mutants [21]. However, it remains unclear whether CPE is indispensable for bacterial cytotoxicity in vitro [14–16].

Recently, the significance of toxins in the induction of in vitro cytotoxicity has been investigated using human intestinal epithelial Caco-2 cells infected with toxin gene-harboring C. perfringens strains and their mutants or anti-toxin antibody [22–24]. Allaart et al. revealed that β2 toxin is not involved in Caco-2 cell cytotoxicity during infection with a cpe2-harboring C. perfringens strain [22]. Agr-like quorum sensing system and VirS/VirR two-component system in a C. perfringens type C isolate are essential for causing in vitro cytotoxicity to Caco-2 cells during infection [23,24]. However, no studies have been conducted on in vitro cell cytotoxicity by C. perfringens type A FP and NFB strains during sporulation.

In this regard, we co-cultured wild-type and cpe-null mutants of C. perfringens FP and NFB strains with Caco-2 cells, and observed cytotoxicity during bacterial sporulation. Our co-culture experiments indicate that the essential toxin (factor) to induce in vitro cytotoxicity by a FP strain SM101 carrying a chromosomal cpe gene is different from that of a NFB strain F4969 carrying a plasmid-borne cpe gene.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. To achieve the sporulation, bacteria were inoculated into fluid thioglycollate (FTG; BD, Franklin Lakes, NJ) medium and incubated anaerobically for 18 h at 37 °C. One milliliter of the bacterial culture was passaged into 10 ml of Duncan-Strong medium [25] and cultured for 24 h at 37 °C. One milliliter of the culture was then heated at 75 °C (strains NCTC8239 and F4969) or 65 °C (strain SM101) for 20 min and passaged into 10 ml of fresh Duncan-Strong medium. The heat treatment and passage were repeated until the amount of spores observed by phase-contrast microscopy was greater than half of the total number of bacteria. These bacterial cells were heated and stored at −80 °C with glycerol for future use.

2.2. Co-culture study

Human colonic Caco-2 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. The cells were plated in a 24-well plate (1.3 × 10^5 cells per well) and incubated for 4 days. Just before the inoculation of bacteria, the cells were washed using phosphate buffered saline (PBS) three times and incubated in 0.65 ml of glucose-negative DMEM (DMEM(−); Life Science Technologies, Carlsbad, CA) supplemented with 0.4% glucose, 0.4% starch, or 0.4% starch containing deoxycholate (DCA; Wako, Osaka, Japan) or ox bile (Wako). The bacterial strains prepared as described above were pre-cultured in FTG anaerobically overnight at 37 °C. The cultures were washed with PBS twice and 65 μl of bacterial culture (1 × 10^9 colony-forming units (CFU) per ml in strain SM101 or 1–5 × 10^2 CFU/ml in other strains) were inoculated into Caco-2 cells prepared as described above and incubated in the CO2 incubator at 37 °C. The turbidity of the cultures was monitored by measuring optical density at 650 nm (OD650) using a spectrophotometer (V630B10; Jasco, Easton, MD). The OD650 for bacterial growth was calculated as follows:

\[ \text{OD}_{650(\text{bacteria})} = \text{OD}_{650(\text{whole})} - \text{OD}_{650(\text{Caco-2 cells})} \]

where OD650 (whole) is an OD650 of cell suspension containing both bacteria and Caco-2 cells, and OD650 (Caco-2 cells) is an OD650 of cell suspension containing only non-infected Caco-2 cells. The number of viable vegetative cells was determined by plating serially diluted samples onto Brain Heart Infusion (BHI) agar, incubating at 37 °C for 24 h in anaerobic conditions, and calculating CFU. The number of heat-resistant spores was counted by plating heat-treated cultures onto BHI agar. The detection threshold was 200 CFU/ml.

2.3. Western blotting

Cultures were centrifuged at 2350× g for 10 min and supernatants (20 μl) were subjected to 12% SDS-PAGE. After transferring to a PVDF membrane, the samples were probed with a rabbit anti-CPE antibody kindly provided by Dr. Yasuhiko Horiguchi (Osaka University, Japan) or a rabbit anti-β-toxin antibody kindly provided by Dr. Masahiro Nagahama (Tokushima Bunri University, Japan) for 1 h at 37 °C. A horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) was then reacted for

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Production of CPE</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>JCM1290</td>
<td>Type A gas gangrene strain</td>
<td>–</td>
<td>JCM^a</td>
</tr>
<tr>
<td>NCTC8239</td>
<td>Type A FP strain, carries chromosomal cpe gene</td>
<td>+</td>
<td>NCTC^b</td>
</tr>
<tr>
<td>WS837</td>
<td>Type A FP strain</td>
<td>+</td>
<td>TMIPH</td>
</tr>
<tr>
<td>W09-505</td>
<td>Type A FP strain</td>
<td>+</td>
<td>TMIPH</td>
</tr>
<tr>
<td>SM101</td>
<td>Electroporatable derivative of type A FP strain NCTC8798, carries chromosomal cpe gene</td>
<td>+</td>
<td>T. Shimizu</td>
</tr>
<tr>
<td>MY15</td>
<td>SM101 cpe::intron</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td>MY15 (pJRC200)</td>
<td>MY15 complemented with pJRC200</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>F4969</td>
<td>NFB disease type A strain, carries plasmid-borne cpe gene</td>
<td>+</td>
<td>[21]</td>
</tr>
<tr>
<td>MR54969</td>
<td>F4969 cpe::catP</td>
<td>–</td>
<td>[21]</td>
</tr>
</tbody>
</table>

^a Japan Collection of Microorganisms. ^b National Collection of Type Cultures. ^c Tokyo Metropolitan Institute of Public Health.
1 h at 37 °C and detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK).

2.4. Caco-2 cell cytotoxicity assay

The LDH cytotoxicity test was performed [26] according to the manufacturer’s instructions (Wako) with slight modifications. Briefly, co-cultures of C. perfringens/Caco-2 cells were centrifuged at 150 × g for 3 min. Supernatant (12.5 μl) was transferred to a 96-well assay plate and diluted with 37.5 μl of distilled water. Samples cultured in DMEM(−) with 0.4% starch and 0.1% Tween 20 were used as positive controls. The supernatants of non-infected Caco-2 cells were used as negative controls. After 50 μl of reactive solution was dispensed into each well, the plate was incubated for 15 min at room temperature. After termination of the reaction with 100 μl of stop solution, the absorbance of each sample was measured at 560 nm in an automated plate reader (ARBO X5; PerkinElmer, Waltham, MA). The cell injury rate was calculated according to the manufacturer’s instructions.

2.5. RNA extraction

Bacterial cultures containing 2 × 107 CFU of C. perfringens were treated with RNAProtect Bacteria Reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After centrifugation at 5000 × g for 10 min, the pellets were washed with SET buffer (25% sucrose, 50 mM EDTA (pH 8.0) and 50 mM Tris–HCl (pH 8.0)) at 5000 × g for 10 min. The pellets, which were suspended in GTC buffer (4 M guanidine thiocyanate, 0.5% Na N-lauryl sarcosine, 25 mM sodium citrate (pH 7.0) and 0.1 M β-mercaptoethanol) [27,28], were homogenized by passing 3 times through a 21-gauge needle to disrupt Caco-2 cell membranes. After being centrifuged at 5000 × g for 10 min, the pellets were washed with SET buffer once. The bacterial cells were lysed by suspending in 100 μl SET buffer with 20 mg/ml lysozyme (Sigma) and 100 μg/ml proteinase K (Roche Applied Science, Upper Bavaria, Germany) at 37 °C for 30 min. Following incubation, they were transferred to a tube containing zirconia beads (Easy Beads; AMR, Gifu, Japan), vortexed for 5 min, and centrifuged at 21,130 × g for 5 min. Total RNA was extracted from the supernatants using TRI reagent LS (Sigma) according to the manufacturer’s instruction.

2.6. Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was digested with DNase I (RQ1 RNase-Free DNase; Promega, Madison, WI) according to the manufacturer’s instructions. cDNA was synthesized using Superscript III reverse transcriptase (Life Science Technologies). Synthesized cDNA was subjected to PCR using Go Taq DNA polymerase (Promega) with gene-specific forward and reverse primer sets (Table 2).

2.7. DNA extraction

Genome DNA from C. perfringens was purified from overnight cultures grown in GAM broth (Nissui, Tokyo, Japan) [29]. Briefly, after 2 ml cultures were centrifuged at 21,130 × g for 5 min, the pellets were suspended in 400 μl TES buffer (50 mM Tris–HCl (pH 8.0), 1 mM EDTA, and 6.7% sucrose) and incubated for 5 min at 37 °C. After addition of 100 μl of 20 mg/ml lysozyme and incubation for 10 min at 37 °C, they were mixed with 160 μl Tris–EDTA buffer (50 mM Tris–HCl (pH 8.0) and 0.25 M EDTA) containing 20% SDS and incubated for 15 min at 37 °C. Then, 20 μg RNase (NipponGene, Tokyo, Japan) was added, and the samples were incubated for 15 min at room temperature. DNA was extracted by phenol:-chloroform:isoamyl alcohol (Nakarai, Kyoto, Japan) and precipitated by isopropanol.

2.8. Southern blotting

Purified DNA samples were digested overnight with HindIII or XbaI at 37 °C according to the manufacturer’s instructions (Takara, Shiga, Japan) and separated by electrophoresis on a 1% agarose gel. The separated DNA digestion products were transferred onto a positively charged nylon membrane (Hybond-N; GE Healthcare) for hybridization with a digoxigenin (DIG)-labeled intron-specific probe, which was prepared as previously described [30] using the primer pair PrMY8 and 9 (Table 2). CSPD substrate (Roche Applied Science) was used to detect probe hybridization according to the manufacturer’s instructions.

2.9. Construction of a C. perfringens strain SM101 cpe-null mutant

The cpe gene of SM101 was inactivated by insertion of a group II intron using the Clostridium-modified TargeTron (Sigma) insertion mutagenesis system [31,32]. Utilizing optimal intron insertion sites identified in the SM101 genome sequence on the Sigma TargeTron website, an intron was targeted in the antisense orientation between nucleotides 195 and 196 of the cpe ORF. The primers used to target the intron to the cpe ORF were PrMY8, 9, 10, and 17 as shown in Table 2. A 350 bp PCR product was inserted into pJIR750ai to construct a cpe-specific TargeTron plasmid. The resultant plasmid, named pMY4, was electroporated into wild-type SM101 by MicroPulser (Bio-Rad, Hercules, CA) (1500 V, 4 ms). Transformants were selected onto BHI agar plates containing 15 μg/ml of chloramphenicol. Bacterial clones carrying an intron insertion were screened by PCR using primers PrMY20, 21, and 3 as shown in Table 2.

### Table 2

<table>
<thead>
<tr>
<th>PrMY4</th>
<th>16S rRNA-F</th>
<th>5′-CGCATATGTTGAAAGATGG-3′</th>
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<tr>
<td>PrMY5</td>
<td>16S rRNA-R</td>
<td>5′-CCTTTAGGCGCTTACC-3′</td>
</tr>
<tr>
<td>PrMY162</td>
<td>pfoA-F</td>
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<td>PrMY163</td>
<td>pfoA-R</td>
<td>5′-CTCCTAATTTTCATTGCGG-3′</td>
</tr>
<tr>
<td>PrMY8</td>
<td>IBS_195/6a</td>
<td>5′-AAAAAGCCTTAATCTTTTCTAGACAGGAGGTGCCC-3′</td>
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<tr>
<td>PrMY9</td>
<td>EBS1d_195/6a</td>
<td>5′-CAGATTGTTCAAATTTTCTGGGATCAAGACATAGGATTAGCTTTC-3′</td>
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<tr>
<td>PrMY10</td>
<td>EBS2_195/6a</td>
<td>5′-TGAACCAAGTTCATTGTAGCTTTCAGAGAAAGTCT-3′</td>
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<tr>
<td>PrMY17</td>
<td>EBSuniversal</td>
<td>5′-CAGAACAGTACTGGCCTGACAGA-3′</td>
</tr>
<tr>
<td>PrMY20</td>
<td>cpe195/6-F</td>
<td>5′-AAGGACATGTTGCGCTTACC-3′</td>
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<tr>
<td>PrMY21</td>
<td>cpe195/6-R</td>
<td>5′-ACCCAGTTGAGGATACATT-3′</td>
</tr>
<tr>
<td>PrMY3</td>
<td>intron-R</td>
<td>5′-GGTTTATGACGATTTAAGGGTG-3′</td>
</tr>
</tbody>
</table>

Ref. [55] [55] [55] [53] [53] [53] [53]
2.10. Construction of complementing strains for cpe-null mutants

The cpe-null mutant MY15 was passaged in BHI broth to cure the plasmid, and confirmed the deletion of the plasmid by sensitivity to chloramphenicol. The cpe expression plasmid pJRC200 [33] was then electroporated into strain MY15 by MicroPulser. Transformants were selected onto BHI agar plates containing 50 µg/ml of erythromycin.

2.11. Reverse passive latex agglutination assay

The assay was performed according to the manufacturer’s information (PET-RPLA; Denka Seiken Co., Ltd, Niigata, Japan). Briefly, co-cultures of C. perfringens/Caco-2 cells were centrifuged at 900 × g for 20 min. The supernatants were serially 4-fold diluted in a 96-well plate, and latex was added. The plate was then incubated at room temperature for 18 h.

2.12. Transepithelial electrical resistance (TER)

Caco-2 cells were seeded on collagen-coated permeable membrane supports (Transwell-COL; Corning Incorporated, Corning, NY) placed in 24-well plates at a density of 7.5 × 10⁴ cells per well, and incubated for 4 days in a CO₂ incubator. C. perfringens were co-cultured with Caco-2 cells as described above but with slight modifications. Bacterial suspension (150 µl) was added to the apical compartment of the Transwell chamber. The TER across the monolayer was measured with Millicell-ERS (Merck, Darmstadt, Germany) [34].

2.13. Statistical analyses

Data are expressed as means ± SD or SEM. Statistical analysis was performed by Student’s or Welch’s t-test. The statistical significance of multiple comparisons was determined by one-way ANOVA, followed by the Tukey–Kramer test. P < 0.05 was considered significant.

3. Results

3.1. Cytotoxicity of C. perfringens strains during vegetative cell growth

C. perfringens is an anaerobic bacterium that generally grows in oxygen-limiting conditions. To create conditions suitable for bacterial growth and its pathogenic effect on host cells in a single experiment, we co-cultured C. perfringens strains (NCTC8239, W5837, or W09-505 derived from FP, or JCM1290 derived from gas gangrene) with human intestinal Caco-2 cells in DMEM(−) containing 0.4% glucose (G/DMEM(−)) in a CO₂ incubator. In the presence of Caco-2 cells, robust bacterial growth was observed with all the strains tested at 8 h post-inoculation (hpi) (Fig. 1A), implying that the metabolism of Caco-2 cells, such as consumption of oxygen and/or secretion of metabolites, is suitable to C. perfringens cell growth. During bacterial growth, sporulation and production of CPE were not observed for all the strains (Figs. S1A and S1B). To measure cytotoxicity, we first confirmed that C. perfringens did not release detectable LDH by using the supernatants of the cultured bacteria in the absence of Caco-2 cells (data not shown). Caco-2 cells were not damaged by the FP strains during the experiment, but the gas gangrene strain JCM1290 showed profound cytotoxicity at 8 hpi (Fig. 1B). These results indicate that the C. perfringens FP strains do not induce cytotoxicity to Caco-2 cells during vegetative cell growth.

3.2. Production of toxins by C. perfringens strains during vegetative cell growth

To understand the mechanism of JCM1290-mediated cytotoxicity during vegetative cell growth, we assessed the production of α-toxin and the expression of pfoA gene encoding perfringolysin O. When C. perfringens isolates were co-cultured with Caco-2 cells in G/DMEM(−), α-toxin was detected in the supernatants of strain JCM1290 at 4 hpi by Western blot analysis (Fig. 1C). In contrast, all tested FP strains did not produce detectable α-toxin. Our PCR survey failed to detect pfoA gene in FP strains tested (Fig. S2), which is consistent with the previous finding that most of the type A chromosomal cpe strains do not carry pfoA [35]. When the pfoA mRNA expression level was determined in strain JCM1290 co-cultured with Caco-2 cells in G/DMEM(−), the expression of pfoA was increased at 4 hpi compared to 0 hpi, and then decreased at 8 hpi (Fig. 1D). No pfoA-specific band was detected in PCR without
reverse transcription (data not shown). These results suggest that the \(\alpha\)-toxin and perfringolysin O are likely to play some role in the Caco-2 cell cytotoxicity by strain JCM1290 during vegetative cell growth.

3.3. Cytotoxicity of a *C. perfringens* FP strain during sporulation

To induce sporulation, we used starch [36] instead of glucose as a carbon source in the co-culture medium because glucose is known to inhibit sporulation [37,38]. A bile salt DCA, previously reported to increase sporulation [39–41], was also used. We confirmed that 0.001–0.1% bile and 0.4–250 \(\mu\)M DCA indeed accelerated sporulation of strain NCTC8239 in our co-culture system (Figs S3A and S3B). When we co-cultured strain NCTC8239 in G/DMEM(−), DMEM(−) containing 0.4% starch (S/DMEM(−)), or DMEM containing 0.4% starch and 50 \(\mu\)M DCA (S/D/DMEM(−)), the turbidity of the cultures increased significantly in the presence of Caco-2 cells (Fig. 2A), indicating that these media supported bacterial growth similarly. As expected, sporulation was not detected in G/DMEM(−) (Fig. 2B). By contrast, sporulation was induced in S/DMEM(−); the number of spores was 1 \(\times\) 10³ and 2 \(\times\) 10⁴ CFU/ml at 8 and 24 hpi, respectively. The addition of DCA further induced sporulation, and the number of spores reached 1 \(\times\) 10⁶ CFU/ml at 8 hpi. Increased production of CPE was also observed in the presence of DCA or bile at 24 hpi (Figs. 2C, S3C, and S3D). We then assessed cytotoxicity quantitatively in Caco-2 cells using the LDH assay. We first confirmed that all the media used in this study were not cytotoxic in the LDH assay in the absence of bacteria (data not shown). Strain NCTC8239 cultured in G/DMEM(−) did not induce cytotoxicity until 24 hpi (Fig. 2D). By contrast, cell death was observed in NCTC8239 cultured in S/DMEM(−), and exacerbated when it was cultured in S/D/DMEM(−) at 24 hpi (Fig. 2D). Addition of bile also increased the cytotoxicity of NCTC8239 (Fig. S3E). Collectively, these results suggest that the cytotoxicity by strain NCTC8239 during co-culturing in S/DMEM(−) and S/D/DMEM(−) was due to the induction of sporulation and production of CPE.

3.4. Cytotoxicity of cpe-null mutant during sporulation

To confirm whether the cytotoxicity of the *C. perfringens* FP strain is mediated by the production of CPE during sporulation, we

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**Fig. 2.** Cytotoxicity of a *C. perfringens* FP strain during sporulation. Strain NCTC8239 was co-cultured with Caco-2 cells in G/DMEM(−) (diamonds), S/DMEM(−) (squares), or S/D/DMEM(−) (triangles) for 8 or 24 h in a CO₂ incubator. (A) The turbidity of NCTC8239 was measured in the presence (solid symbols) or absence (open symbols) of Caco-2 cells. **P < 0.01 compared to absence of Caco-2 cells. Data represent the means ± SD of three independent experiments. (B) The number of vegetative cells (solid symbols) or spores (open symbols) was determined by plating serially diluted unheated (for vegetative cells) or heat-treated (for heat-resistant spores) samples, respectively, onto BHI agar, incubating at 37 °C for 24 h in anaerobic conditions and counting CFU. Data represent the means ± SEM of three independent experiments. (C) The presence of CPE in the supernatants of the co-cultures at 24 hpi was assessed by Western blot analyses using a CPE-specific antibody. M indicates molecular size marker. (D) The cytotoxicity of Caco-2 cells was measured by LDH assay. *: P < 0.05 compared to G/DMEM(−). Data represent the means ± SEM of three independent experiments.

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**Fig. 3.** Construction of cpe-null mutant of strain SM101. (A) A schematic diagram showing the location of intron insertion, primer target regions (PrMY20, 21, and 22) used in B, and recognition sites of HindIII and XbaI. (B) A single colony from wild-type SM101, cpe mutant MY15, and complemented MY15(pJRC200) was subjected to PCR using the cpe-specific primers PrMY20 and PrMY21 (upper panel) or cpe-intron primers PrMY20 and PrMY3 (lower panel). M indicates molecular size marker. (C) Southern hybridization analysis for the presence of a group II intron insertion in SM101, and MY15. Genomic DNA from each strain was digested with HindIII or XbaI, and electrophoresed on a 1% agarose gel. After transferred onto a nylon membrane, it was hybridized with a DIG-labeled intron-specific probe. (D) SM101, MY15, and MY15(pJRC200) were co-cultured with Caco-2 cells in S/D/DMEM(−) for 36 h. Culture supernatants were subjected to Western blot analyses using a CPE-specific antibody.
introduced a cpe-null mutation in strain SM101 (a derivative of FP strain NCTC8798) using the Targetron method [11]. The mutator plasmid pMY4 designed to insert intron to the cpe gene (Fig. 3A) was introduced into SM101 by electroporation and the cpe-null mutant strain MY15 was isolated as previously described [32]. The PCR analyses confirmed the insertion of intron into the cpe gene in MY15 (Fig. 3B). Southern blot analysis using an intron-specific probe demonstrated the presence of a single intron insertion into the genome of the cpe-null mutant MY15 (Fig. 3C). Complemented MY15 was prepared by introducing the recombinant plasmid pJRC200 [33] carrying wild-type cpe into MY15 by electroporation. The presence of wild-type cpe (Fig. 3B, upper panel) and the insertion of intron (Fig. 3B, lower panel) were confirmed in MY15(pJRC200) by the PCR analysis. The fragment with an intron insertion (1.1 kb band in Fig. 3B, upper panel) was not detected in MY15(pJRC200), suggesting that multiple copies of the cpe gene of plasmid origin, whose amplification far outweighs that of the mutated chromosomal cpe, are present in MY15(pJRC200). When strains SM101, MY15, and MY15(pJRC200) were co-cultured with Caco-2 cells in S/D/DMEM(−) for 36 h, CPE was detected in the culture supernatants of SM101 and MY15(pJRC200), but not with MY15, by Western blot analysis (Fig. 3D). These results indicated that strain MY15 is defective in CPE production and this defect is restored in the complemented strain MY15(pJRC200).

We then co-cultured SM101, MY15, or MY15(pJRC200) with Caco-2 cells in S/D/DMEM(−) and assessed bacterial growth and sporulation. Strains SM101 and MY15 showed similar kinetics in their growth and sporulation: the numbers of vegetative and sporulating cells each reached $1 \times 10^6$ CFU/ml at 12 hpi and then gradually decreased until 36 hpi (Fig. 4A). CPE was detected at 12 hpi in the culture supernatants of SM101 by Western blot analysis (Fig. 4B). As expected, MY15 did not produce CPE during the co-culture experiment. When MY15(pJRC200) was co-cultured with Caco-2 cells at $1 \times 10^5$ CFU/ml (the equivalent titer to SM101 and MY15), it showed lower bacterial growth ($1 \times 10^5$ CFU/ml at 12 hpi) and sporulation ($1 \times 10^5$ CFU/ml at 24 hpi) compared to SM101 (Fig. 5A). MY15(pJRC200) restored the production of CPE but it was detectable at 36 hpi (Fig. 5B). In order to detect enough amounts of spores and CPE in the cultures of MY15(pJRC200), we co-cultured MY15(pJRC200) at $1 \times 10^5$ CFU/ml. In those, the number of spore cells reached $1 \times 10^6$ CFU/ml and the detectable amount of CPE was restored at 12 hpi (Fig. 5A and B). Therefore, MY15(pJRC200) was co-cultured at $1 \times 10^5$ CFU/ml for further research. To measure the concentration of CPE released in the culture, we performed Western blot analysis using the 20 μl supernatant of SM101 at 12 hpi and compared the intensity of the band of CPE to those in 1 to 1000 ng CPE (Fig. 4C). The intensity of the band of CPE in SM101 was equivalent to that in 100 ng CPE. Therefore the amount of CPE in the supernatants of SM101 was estimated to be approximately 5 μg/ml CPE at 12 hpi. We next employed a reverse passive latex agglutination assay to detect CPE at 3, 6, 9, and 12 hpi with SM101 (Fig. 4D) because it was predictable that the amount of CPE produced was not detectable in Western blot analysis in the earlier phase of co-culture. The latex agglutination titers were between 2 and 8 in the supernatants of 3 and 6 hpi, and increased to 512 to 8192 at 9 and 12 hpi. The titers at 12 hpi were equivalent to those of controls: 10 μg/ml CPE. These results indicate that the amount of CPE gets increased in the supernatants at 6 to 9 hpi and reaches 5–10 μg/ml at 12 hpi in the co-culture condition.

We next assessed the cytotoxicity on Caco-2 cells by these strains in C. perfringens/Caco-2 co-cultures at 12, 24, and 36 hpi by phase-contrast microscopy (Fig. 5A). Caco-2 cells co-cultured with SM101 showed cell rounding and detachment at 12 hpi. By contrast, MY15 did not show any cytotoxic effect until 36 hpi. MY15(pJRC200) restored cytotoxicity, and Caco-2 cells were damaged at 12 hpi as in SM101. Consistent with these observations, LDH release from the cells significantly increased with SM101 at 12 hpi; the rate of cytotoxicity was approximately 50% at 12 hpi and reached 80% at 24 hpi (Fig. 5B). Cytotoxicity was not detected with MY15 until 24 hpi and then slightly increased at 48 hpi. As expected, MY15(pJRC200) restored LDH release, and the cytotoxicity reached similar levels as that of SM101 at 12 hpi. We also assessed the epithelial cell integrity by measuring the TER [42] across the Caco-2 monolayer (Fig. 5C) because CPE is known to bind claudins, components of tight junctions, and disrupt tight junction barriers [43]. The TER decreased in the SM101 co-culture at 4 hpi and significantly reduced until 10 hpi. By contrast, MY15 did not induce any TER decrease during the experiment, as observed with non-infected cells. MY15(pJRC200) restored the significant decrease in TER. MY15(pJRC200) co-cultured at $1 \times 10^4$ CFU/ml also showed cytotoxicity by the LDH assay at 36 hpi (Fig. 5C). The delay of cytotoxicity is consistent with the delayed release of CPE (Figs 5A and 5B).

On the other hand, MY15(pJRC200) co-cultured at $1 \times 10^5$ CFU/ml showed remarkable decrease in the TER as that in SM101 (Fig. 5D), suggesting that the TER is more sensitive assay to assess Caco-2 cell damage than the LDH assay. Collectively, C. perfringens FP strain SM101 shows a cytotoxicity to Caco-2 cells that is exclusively dependent on the production of CPE during sporulation.

3.5. Cytotoxicity of a C. perfringens NFB strain

Most type A FP strains encode the cpe gene in their
chromosome, whereas NFB strains typically carry a plasmid-borne cpe gene. We therefore examined whether a C. perfringens NFB strain F4969 carrying a plasmid-borne cpe gene shows CPE-dependent cytotoxicity on Caco-2 cells like FP strain SM101. First, we co-cultured strain F4969 or its cpe-null mutant MRS4969 with Caco-2 cells in G/DMEM(−) or S/D/DMEM(−), and assessed bacterial cell growth and sporulation (Fig. 6A). The number of vegetative cells was approximately 1 \times 10^6 CFU/ml at 8 and 24 hpi in both strains cultured in G/DMEM(−) or S/D/DMEM(−). As expected, no sporulating cells were detected in strain F4969 cultured in G/DMEM(−). By contrast, strains F4969 and MRS4969 sporulated similarly in S/D/DMEM(−); the number of spores reached approximately 1 \times 10^5 CFU/ml at 8 hpi. We next examined cytotoxicity of the strains (Fig. 6B). LDH release occurred at 8 hpi in strain F4969 cultured in G/DMEM(−). We also observed cytotoxicity of strain F4969 during sporulation in S/D/DMEM(−) at 8 hpi. It is noteworthy that the LDH release was observed with cpe-null mutant strain MRS4969 similarly to wild-type F4969 during sporulation at 8 and 24 hpi. In order to dissect the role of toxins for cytotoxicity by strain F4969, we assessed the production of CPE and α-toxin, and the expression of pfoA gene. When the supernatants of the strains co-cultured with Caco-2 cells were subjected to Western blot analysis, CPE was not detected in F4969 cultured in G/DMEM(−) during the experiment (Fig. 6C, upper panel). During sporulation in S/D/DMEM(−), detectable CPE was produced at 24 hpi by strain F4969 but not strain MRS4969 as expected. In Western blot analysis, both strains F4969 and MRS4969 produced detectable amount of α-toxin at 8 and 24 hpi in S/D/DMEM(−) (Fig. 6C, lower panel). By contrast, α-toxin was detected slightly at 8 hpi in F4969 in G/DMEM(−). Finally when we examined the expression levels of the pfoA gene by RT-PCR (Fig. 6D), strain F4969 cultured in both G/DMEM(−) and S/D/DMEM(−) expressed the pfoA gene higher at 4 and 8 hpi than 0 hpi. Strain MRS4969 also increased the expression level of the pfoA gene at 4 hpi compared to 0 hpi. No pfoA-specific band was detected in PCR without reverse transcription (data not shown). Collectively, the results suggest that the Caco-2 cell cytotoxicity caused by the NFB strain F4969 is not dependent on CPE production, and perhaps other toxin(s) plays a major role to induce this cytotoxicity.

4. Discussion

Previous in vivo study demonstrated that CPE expression is both necessary and sufficient for C. perfringens type A disease isolates SM101 and F4969 to induce histopathological damage in rabbit ileal...
loop model [21]. In vitro, purified CPE has shown host cell cytotoxicity by forming pores on the surface of cell membrane [12,14–16]. However, it is still unknown whether CPE is indispensable for in vitro host cell cytotoxicity. In the current study, we demonstrated that CPE is essential to induce in vitro cytotoxicity caused by C. perfringens [21] harboring a chromosomal cpe by co-culturing cpe-null mutants with Caco-2 cells. By contrast, CPE does not play a major role to induce in vitro cytotoxicity by NFB strain F4969 carrying a plasmid-encoded cpe, as cpe-null mutant derivative of F4969 (strain MRS4969) showed cytotoxicity to Caco-2 cells during co-culture. It was unexpected because cell lysates of both strains, SM101 and F4969, caused CPE-dependent fluid accumulation and histopathological damage in a rabbit ileal loop model [21]. These discrepancies suggest that some additional toxins might be responsible for F4969-mediated in vitro cytotoxicity. Sayeed et al. showed that perfringolysin O and α-toxin are not essential for the intestinal virulence of C. perfringens type C isolate CN3685 in a rabbit ileal loop model using the cpb, pfoA, or plc-null mutants [44]. On the other hand, perfringolysin O was reported to be the most responsible toxin for the C. perfringens strain 13-dependent in vitro cytotoxicity [45]. Indeed, our current study demonstrated that during co-culturing with Caco-2 cells, (i) NFB strain F4969 expressed the pfoA mRNA (Fig. 5D), whereas FP strains do not even carry the pfoA gene (Fig. 52); and (ii) strain F4969 produced detectable level of α-toxin in the sporulation condition (Fig. 6C). Thus, perfringolysin O and α-toxin (and perhaps other toxins) are likely to be more important than CPE for in vitro cytotoxicity induced by strain F4969. However, it is still unclear (i) which bacterial factor(s) is essential to induce in vitro cytotoxicity in strain F4969, (ii) whether most, if not all, isolates derived from FP or NFB diseases reproduce the results for in vitro cytotoxicity shown in this study, and (iii) whether the difference in in vitro cytotoxicity between a FP and NFB strain influences in vivo pathogenicity, even though rabbit ileal loop model revealed that CPE is an essential factor for the GI damage [21]. Further studies employing a large number of FP and NFB strains and specific gene-deficient isogenic mutants should clarify these unanswered questions.

The higher production of α-toxin by a gas gangrene strain JCM1290 during co-culture, compared to FP strains (Fig. 1C), suggested that the regulation of α-toxin production in strain JCM1290 might be different from that in the tested FP strains. plc expression is positively regulated by VirR/VirS through VR-RNA [46,47]. Recently, Obana et al. reported that VR-RNA matured and activates mRNA of colA, another toxin regulated by VirR/VirS/VR-RNA, by pairing to the sequence of 5′UTR of colA [48,49]. Also, the production of α-toxin is partly dependent on the promoter sequence of plc [50]. Indeed, the promoter sequence of plc in strain NCTC8237 is identical with that of strain 13 that produces intermediate level of α-toxin. In contrast, strain JCM1290 plc promoter is identical to that of strain NCTC8237 producing high level of α-toxin. Recently, Katayama et al. described that the specific affinity of the C. perfringens polymerase subunits for the phased A-tracts upstream of the plc promoter is likely to contribute to gene expression [51]. Although the molecular mechanism of the regulation of toxin genes is not fully understood in C. perfringens, it is possible that the promoter sequences of toxin genes and the affinity of regulatory factors and polymersases to the binding regions would influence to the abilities of toxin production, leading to different Caco-2 cell cytotoxicity in C. perfringens.

In the present study, we developed a C. perfringens/Caco-2 co-culture system where the anaerobic C. perfringens grew and exerted its pathogenic effects on host cells in a CO₂ incubator. This model system enables us to obtain reproducible results without any laborious regulation of oxygen content in the culture conditions. Modification of medium constituents is also easy, which allows us to control the growth condition for vegetative and sporulating cells. Indeed, C. perfringens cells grew to a level of 10⁵ CFU/ml, formed spores extensively, and produced several micrograms of CPE in the presence of Caco-2 cells in a single culture well. Also 5–10 µg/ml of CPE released during sporulation was sufficient to cause cytotoxicity by strain SM101 in this system. This is consistent with the previous reports in which 10–50 µg/ml of CPE caused fluid accumulation and damage to intestinal villi in animal intestinal loop models and human ileum tissues [18–20,32]. Recent studies revealed host–pathogen cross-talk between C. perfringens and the cultured host cells including Caco-2 cells; C. perfringens vegetative cells significantly up-regulate the production of many toxins when grown in the presence of cultured Caco-2 cells [53], and Agr-like quorum sensing and VirS/VirR systems participate in this signaling [23,24,54]. Although it has not yet been determined whether host–pathogen cross-talk is also applicable for CPE positive type A strains, our in vitro co-culture model system can be used to assess the complicated interactions between environmental factors, bacterial cells, and host cells that usually take place within patients' intestinal tracts [9].
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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micpath.2015.04.003.

References


