Bystander Host Cell Killing Effects of *Clostridium perfringens* Enterotoxin

Archana Shrestha, Matthew R. Hendricks, Jennifer M. Bomberger, Bruce A. McClane

Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

**ABSTRACT** *Clostridium perfringens* enterotoxin (CPE) binds to claudin receptors, e.g., claudin-4, and then forms a pore that triggers cell death. Pure cultures of host cells that do not express claudin receptors, e.g., fibroblasts, are unaffected by pathophysiologically relevant CPE concentrations in vitro. However, both CPE-insensitive and CPE-sensitive host cells are present in vivo. Therefore, this study tested whether CPE treatment might affect fibroblasts when cocultured with CPE-sensitive claudin-4 fibroblast transfectants or Caco-2 cells. Under these conditions, immunofluorescence microscopy detected increased death of fibroblasts. This cytotoxic effect involved release of a toxic factor from the dying CPE-sensitive cells, since it could be reproduced using culture supernatants from CPE-treated sensitive cells. Supernatants from CPE-treated sensitive cells, particularly Caco-2 cells, were found to contain high levels of membrane vesicles, often containing a CPE species. However, most cytotoxic activity remained in those supernatants even after membrane vesicle depletion, and CPE was not detected in fibroblasts treated with supernatants from CPE-treated sensitive cells. Instead, characterization studies suggest that a major cytotoxic factor present in supernatants from CPE-treated sensitive cells may be a 10- to 30-kDa host serine protease or require the action of that host serine protease. Induction of caspase-3-mediated apoptosis was found to be important for triggering release of the cytotoxic factor(s) from CPE-treated sensitive cells. Furthermore, the cytotoxic factor(s) in these supernatants was shown to induce a caspase-3-mediated killing of fibroblasts. This bystander killing effect due to release of cytotoxic factors from CPE-treated sensitive cells could contribute to CPE-mediated disease.

**IMPORTANCE** In susceptible host cells, *Clostridium perfringens* enterotoxin (CPE) binds to claudin receptors and then forms pores that result in cell death. Using cocultures of CPE receptor-expressing sensitive cells mixed with CPE-insensitive cells lacking receptors for this toxin, the current study determined that CPE-treated sensitive cells release soluble cytotoxic factors, one of which may be a 10- to 30-kDa host serine protease, to cause apoptotic death of cells that are themselves CPE insensitive. These findings suggest a novel bystander killing mechanism by which a pore-forming toxin may extend its damage to affect cells not directly responsive to that toxin. If confirmed to occur in vivo by future studies, this bystander killing effect may have significance during CPE-mediated disease and could impact the translational use of CPE for purposes such as cancer therapy.

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*Clostridium perfringens* enterotoxin (CPE) is a 35-kDa single polypeptide that lacks significant primary amino acid sequence homology with other toxins (1), but structurally it belongs to the aerolysin pore-forming toxin family (2–4). CPE causes the gastrointestinal symptoms of *C. perfringens* type A food poisoning, which is the second most common bacterial foodborne illness (1, 5, 6) in the United States, where it affects ~1 million people/year (7). Similarly, CPE production is necessary for *C. perfringens* type A strains to cause ~5 to 10% of all human nonfoodborne gastrointestinal disease cases (6, 8). This toxin may also contribute to some human nonfoodborne gastrointestinal disease cases (6, 8).

CPE action begins when this toxin binds to claudin receptors on host cells. Claudins, a large family of proteins that typically have a mass of ~20 to 27 kDa, are important mammalian tight junction components (10). Some claudins (e.g., claudin-1) bind CPE poorly or not at all, while other claudins are receptors with strong (e.g., claudin-3 or -4) or moderate (e.g., claudin-8 or -14) CPE binding affinity (11–15).

Once bound to a claudin receptor, CPE becomes sequestered in an ~90-kDa small complex on the host cell surface (16). Those small CPE complexes then rapidly oligomerize into an ~450-kDa prepore containing ~6 CPE molecules (17, 18, 19). When each CPE in the prepore extends a β-hairpin loop, this results in formation of a β-barrel pore in plasma membranes (20). This pore (named CH-1 [19]) allows rapid Ca²⁺ influx into the host cell cytoplasm (21–23). At high CPE doses, a massive calcium influx causes strong calpain activation and host cells die via a form of necrosis known as oncosis (23, 24). At lower CPE doses, where there is less calcium influx and calpain activation, a classical caspase-3/7-mediated apoptosis...
develops (23, 24). Enterocyte cell death leads to intestinal damage and increased fluid and ion secretion (25–27).

Pure cultures of mammalian cells that do not produce claudin receptors are insensitive to pathophysiologically relevant CPE concentrations (15). However, both CPE-sensitive cells and CPE-insensitive cells are present in vivo. Therefore, this study evaluated whether CPE might affect insensitive cells in coculture with sensitive cells.

RESULTS

CPE treatment affects the viability of parent cells cocultured with claudin-4 transfectant cells. Before testing whether CPE treatment might damage naturally CPE-insensitive parent cells in coculture with CPE-sensitive cells, control experiments first demonstrated that (i) 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) staining did not itself affect the viability of parent cells and (ii) pure cultures of those CFSE-stained parent cells remained insensitive to CPE (Fig. 1A and B).

In contrast, CFSE-stained parent cells lost significant viability when treated with CPE in coculture with CPE-sensitive claudin-4 transfectant cells (Fig. 1A and B). After subtracting the nonviable parent cell background (no CPE treatment) always present in coculture with claudin-4 transfectants, ~10% of parent cells were rendered nonviable by a 0.5-μg ml⁻¹ CPE dose in this coculture. This result was significantly higher than the <1% of nonviable cells detected after similar CPE treatment of a pure culture of parent cells.

Supernatants collected from CPE-treated sensitive cell cultures reduce parent cell viability. The CPE-induced parent cell cytotoxicity detected in Fig. 1 could involve a factor(s) released from CPE-treated claudin-4 transfectant cells. If this is the case, then pure cultures of parent cells should exhibit less viability when treated with supernatants collected from similarly CPE-challenged pure parent cell cultures. This hypothesis was verified (Fig. 2A) using the Fig. 1 microscopy approach and confirmed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) cytotoxicity assays (not shown). The release of cytotoxic factor(s) into culture supernatants is not restricted to CPE-sensitive claudin-4 transfectants,
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FIG 2 Culture supernatants from CPE-treated sensitive cells induce cytotoxicity in pure cultures of parent cells. (A) Cytotoxic effects on parent cells of supernatants collected from claudin-4 transfectant cells (Cldn4+CPE sup) or supernatants collected from parent cells (Parent cells+CPE sup) that had been treated for 1 h with 0.5 μg ml⁻¹ of CPE, as measured by fluorescence microscopy. (B) Cytotoxic effects on parent cells of supernatants collected from Caco-2 cells that had been treated for 1 h with 1 μg ml⁻¹ of CPE (+CPE) or without CPE (-CPE), as measured by the MTT assay. Results shown are the means of three repetitions; error bars represent the standard errors of the means. Values that are significantly different (P < 0.05) are indicated by a bar and asterisk.

since this effect was also observed using supernatants from human CPE-treated Caco-2 cells, which are pathophysiologically relevant enterocyte-like cells (Fig. 2B).

The Fig. 1 and 2 experiments used low CPE doses to minimize cell detachment (as necessary for microscopy analysis), so a CPE dose-response experiment was performed with an MTT assay (Fig. 3A, left panel). This analysis showed that CPE dosage affects the extent of parent cell cytotoxicity caused by supernatants collected from claudin-4 transfectant cells. This cytotoxicity was not a direct effect of high CPE doses on parent cell viability, since adding the same CPE doses to supernatants collected from control claudin-4 transfectant cell cultures (no CPE treatment) had no effect on parent cell viability (Fig. 3A, right panel). Last, while the cytotoxic effects of supernatants collected from CPE-treated claudin-4 transfectant cells on parent cells were significant, they were less than those observed in claudin-4 transfectant cells treated directly with comparable CPE amounts (Fig. 3A and B, left panels). As expected, parent cells treated directly with the same high CPE doses showed no altered viability (Fig. 3B, right panel).

Initial characterization of release of cytotoxic factor(s). To begin characterizing release of cytotoxic factor(s) by CPE-treated claudin-4 transfectant cells, we asked whether this effect requires CPE action. Preincubating the toxin with a CPE-neutralizing monoclonal antibody blocked cytotoxic factor release from claudin-4 transfectant cells (Fig. 4A, left panel); this effect was specific, since similar preincubation using a non-CPE-neutralizing monoclonal antibody did not affect cytotoxic factor release from claudin-4 transfectant cells. The importance of fully active CPE for inducing the release of cytotoxic activity from CPE-treated sensitive cells was confirmed when cytotoxic factor release from claudin-4 transfectant cells was detected (Fig. 4A, right panel) after treatment of those cells with native CPE or recombinant CPE (rCPE), but not after similar treatment with rCPE-D48A (rCPE with D48A change), which binds and forms a small complex but cannot form any CH-1 pores (26).

Additional characterization studies suggested the cytotoxic activity of supernatants collected from CPE-treated claudin-4 transfectant cell cultures may involve a heat-labile protein. The cytotoxic activity in those supernatants was reduced by heating (Fig. 4B, left panel) or trypsin treatment (Fig. 4C, left panel). Interestingly, supernatant cytotoxic activity was also impaired by the addition of trypsin inhibitor (Fig. 4C, left panel). The results of ultrafiltration experiments suggested that the cytotoxic factor(s) in crude supernatants has a molecular mass of >10 kDa (Fig. 4D, left panel).

Evaluating whether CPE species are the supernatant cytotoxic factor affecting parent cells. CPE can cause cell lysis and bleb formation in sensitive cells (21, 28), which might release...
receptor-bound CPE monomer and/or the CH-1 pore complex into supernatants of CPE-treated sensitive cells, possibly allowing those CPE species to be taken up by, and then kill, CPE-insensitive parent cells. This possibility was assessed by evaluating the presence of CPE species in supernatants collected from CPE-treated claudin-4 transfectant cells or CPE-treated Caco-2 cells (Fig. 5A and B). CPE Western blotting did detect considerable amounts of CPE monomer in these supernatants, at least some of which is likely to be free toxin that had not bound to the sensitive cells during CPE treatment. While standard Western blot conditions did not detect CH-1 in supernatants collected from CPE-treated claudin-4 transfectant cells or Caco-2 cells, small amounts of CH-1 were detected on long chemiluminescence exposure of the Western blot (not shown), particularly using supernatants from CPE-treated Caco-2 cells.

Differential centrifugation was performed to evaluate whether Caco-2 cells or claudin-4 transfectant cells release extracellular membrane vesicles after CPE treatment. Protein analyses of the obtained pellets detected small (i.e., vesicles pelleted at 100,000 g) and large (vesicles pelleted at 10,000 g) membrane vesicles in supernatants collected from both CPE-treated sensitive cell lines (Fig. 6A), although significantly more membrane vesicles were present in supernatants from CPE-treated Caco-2 cells. The presence of host membrane proteins in pellet fractions containing membrane vesicles released from CPE-treated Caco-2 cells was confirmed by a cadherin Western blot (Fig. 6B); no attempt was made to detect host proteins in CPE-treated claudin-4 transfectant cells due to lower protein levels released by those cells. Compared to supernatants from control Caco-2 or claudin-4 transfectant cells, 10- to 20-fold-more membrane vesicles were present in

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**FIG 4** Characterization of release of cytotoxic factor(s) from CPE-treated sensitive cells. (A) Active CPE (1 μg ml⁻¹) is needed for cytotoxic factor release from CPE-treated sensitive cells. (Left) Samples (x axis) included CPE alone, CPE with MAb3 antibody, CPE with MAb10 antibody, buffer alone, MAb3 alone, or MAb10 alone. The samples were preincubated for 1 h before being applied to claudin-4 transfectant cells for 1 h. Supernatants were collected from those cells, briefly centrifuged at 2,000 x g, and then applied to parent cells for 1 h before cytotoxicity testing using the MTT assay. (Right) rCPE-D48A, rCPE, or native CPE (1 μg ml⁻¹) was added to claudin-4 transfectant cells for 1 h. Supernatants from those cultures were then applied to parent cells for 1 h, and an MTT assay was performed. (B to D) Sensitivity and size analyses of the cytotoxic factor(s) released from claudin-4 transfectant cells. Samples characterized included crude supernatants from claudin-4 transfectant cells treated for 1 h with 0.5 μg ml⁻¹ of CPE (left graphs) or supernatant fractions that were collected from claudin-4 transfectant cells treated with 5 μg ml⁻¹ and then depleted of membrane vesicles by centrifugation at 100,000 x g (right graphs for panels B and C and the middle graph for panel D). (B) Heat sensitivity. Cytotoxic activity remaining in crude supernatants or supernatants depleted of membrane vesicles before and after heating at 70°C for 10 min. (C) Protease sensitivity of cytotoxic factors in crude supernatants or supernatants depleted of membrane vesicles was assessed as follows: (i) supernatant alone (No treatment), (ii) supernatant treated with 200 μg trypsin inhibitor (Inhibitor), (iii) supernatant treated with 100 μg trypsin, followed by the addition of 200 μg trypsin inhibitor (Trypsin + Inhibitor), and (iv) supernatant treated with 100 μg trypsin (Trypsin). (D) Size of cytotoxic factor(s) in crude supernatants that were collected from CPE-treated claudin-4 transfectant cells and then depleted of membrane vesicles. Crude supernatants (left) or membrane-depleted supernatants (middle) were filtered through a 10,000-molecular-weight-cutoff Amicon filter. (Right) Cytotoxic activity of membrane-depleted supernatant filtered through a 30-kDa-cutoff filter, with or without the addition of 200 μg ml⁻¹ trypsin inhibitor to the flowthrough. All samples in panels B to D were applied to parent cells for 1 h before an MTT assay was performed to determine cytotoxicity. Results shown in panels A to D are means from three independent experiments. Error bars represent the standard errors of the means. Values that are significantly different (P < 0.05) are indicated by a bar and asterisk.
supernatants collected from sensitive cells after CPE treatment (Fig. 6A).

CPE Western blot analyses were performed to detect CPE in centrifuge fractions collected from CPE-treated sensitive cells (Fig. 6C). Those analyses revealed that CPE monomer was associated with both the large and small membrane vesicles present in supernatants collected from CPE-treated Caco-2 cells and claudin-4 transfectants. In contrast, CH-1 was associated only with vesicles collected from CPE-treated Caco-2 cells. If membrane vesicle-mediated delivery of CH-1 or bound CPE monomer caused the decreased viability in parent cells treated with supernatants from CPE-treated sensitive cells, CPE species should be present in those parent cells. However, Western blot analyses (Fig. 5A and B) detected no CPE in parent cells challenged with supernatants collected from CPE-treated claudin-4 transfectant cells or Caco-2 cells, even using supernatants from sensitive cells challenged with a high (5 μg ml⁻¹) CPE dose. The absence of CPE species in the supernatant-treated parent cells was not a CPE Western blot insensitivity issue, since CH-1 complex was readily detected in sensitive cells treated directly with 0.5 μg ml⁻¹ of CPE (Fig. 5A and B); that result is informative, since those sensitive cells treated with 0.5 μg ml⁻¹ of CPE exhibited cytotoxicity equivalent to that of parent cells challenged with supernatants collected from sensitive cells treated with 5 μg ml⁻¹ of CPE.

Effects of extracellular membrane vesicle depletion on cytotoxic activity in supernatants collected from CPE-treated sensitive cells. Even if extracellular membrane vesicles were not bringing CPE into parent cells, their presence in supernatants collected from CPE-treated claudin-4 transfectant cells or Caco-2 cells was informative, since CPE species were not present in those parent cells treated with supernatants from CPE-treated sensitive cells.

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vesicle depletion. Although significantly less, some cytotoxicity was also detected in parent cells treated with natural concentrations of resuspended membrane vesicles collected from supernatants of those CPE-treated sensitive cells. In contrast, no significant cytotoxicity was detected (data not shown) in parent cells treated with membrane vesicle-depleted supernatants or natural concentrations of membrane vesicles collected from supernatants of control Caco-2 or claudin-4 transfectant cells.

Characterization of cytotoxic factor activity remaining in extracellular membrane vesicle-depleted supernatants. Since most cytotoxic activity in crude supernatants collected from CPE-treated sensitive cells remained after depletion of extracellular membrane vesicles by a 100,000 g centrifugation, the cytotoxic factor(s) in those fractions was characterized. These studies (Fig. 4A to D, right panels) showed that the cytotoxic factor(s) present in these membrane vesicle-depleted supernatants was heat labile, with an apparent size of 10 to 30 kDa and sensitive to trypsin. As also noted for crude supernatants, the addition of a serine protease inhibitor significantly reduced the cytotoxic effects of these membrane-depleted supernatants (Fig. 4D, right panel).

Supernatants collected from CPE-treated sensitive cells induce caspase-3-mediated apoptotic death in parent cells. The final experiments of this study investigated the cell death mechanism triggered when parent cells were treated with supernatants collected from CPE-treated sensitive cells. This analysis first determined (not shown) that treatment with 1 μg ml⁻¹ of CPE induces caspase-3 activation in claudin-4 transfectant cells and, as reported previously (23), Caco-2 cells. This caspase-3/7 activation was important for CPE-induced death of these cells, since cytotoxicity was blocked by the caspase-3/7 inhibitor N-acetyl-Asp-Glu-Val-Asp-al (Ac-DEVD-CHO) (not shown). The cytotoxic activity in crude supernatants collected from cultures of CPE-treated Caco-2 cells or claudin-4 transfectant cells was dependent upon this caspase 3/7 activation in CPE-sensitive cells. Specifically, pretreating Caco-2 cells or claudin-4 transfectant cells with the caspase 3/7 inhibitor prior to CPE challenge prevented those cells from releasing cytotoxic factors (Fig. 8A), although it did not reduce CPE-induced release of membrane vesicles (Fig. 6A).

Significant caspase-3/7 activity was present in culture supernatants collected from CPE-treated Caco-2 cells or claudin-4 transfectant cells, but not control (no CPE treatment) cells (not shown). However, while direct addition of Ac-DEVD-CHO eliminated caspase 3/7 activity of supernatants collected from CPE-treated Caco-2 cells or claudin-4 transfectant cells, it did not affect the ability of those supernatants to cause cytotoxicity in parent cells (not shown).

The final experiment of this study pretreated parent cells with the caspase-3/7-specific inhibitor. This pretreatment blocked the development of cytotoxicity in those parent cells when they were subsequently treated with supernatants collected from CPE-treated Caco-2 cells or claudin-4 transfectant cells (Fig. 8B).

DISCUSSION

To our knowledge, the current study provides the first indication that supernatants collected from a pore-forming toxin-treated sensitive cell can kill other cells that are, in pure culture, insensitive to that toxin. This bystander killing effect clearly required the pore-forming activity of CPE since (i) it could be blocked by a CPE-neutralizing monoclonal antibody and (ii) sensitive cells treated with rCPE-D48A, which binds and forms small CPE complex, did not induce release of the cytotoxic factor from these cells. It will be of future interest to evaluate whether a toxin-induced bystander killing phenomenon is common to other pore-forming toxins beyond CPE.

The appearance of membrane blebs on CPE-treated sensitive cells was first noted in 1979 (21, 28). The current study now demonstrates that CPE-treated sensitive cells dying from apoptosis not only form membrane blebs but also release high levels of both small and large membrane vesicles into supernatants. CPE can kill sensitive cells by apoptosis (23, 24; this study), and other studies have shown that membrane blebs carrying cytoplasmic contents can be released from apoptotic cells as apoptotic bodies (30). However, pretreatment of Caco-2 cells or claudin-4 transfectant cells with a caspase-3/7 inhibitor did not affect their release of membrane vesicles upon CPE challenge, strongly suggesting that
apoptosis is not required for the release of membrane vesicles from CPE-treated sensitive cells.

The current study also determined that the membrane vesicles released from CPE-treated sensitive cells contain CPE and, for CPE-treated Caco-2 cells, the CH-1 pore. Membrane vesicles released from bacteria can serve as a mechanism for delivering toxins into host cells (31–33), and membrane vesicles released from host cells have been shown to deliver anthrax lethal factor into mammalian cells (34). Therefore, it was conceivable that CPE-containing membrane vesicles might similarly deliver bound CPE monomer or CH-1 into naturally insensitive host cells to create cytotoxic pores. However, no CPE species were detected in parent cells treated with crude supernatants from CPE-treated sensitive cells, even though those supernatants possessed membrane vesicles containing CPE species. Nonetheless, membrane vesicle delivery of CPE species could occur in vivo, for example, so further investigation of this potential phenomenon may be warranted.

Although membrane vesicles released from CPE-treated sensitive cells do not introduce CPE into parent cells, they still might deliver toxic host factors into those cells, e.g., the presence of caspase-3, a potent protease, has been detected previously in exosomes (35). Therefore, we tested whether supernatants collected from CPE-treated sensitive cells remained cytotoxic when depleted of membrane vesicles. However, the majority of cytotoxic activity in crude supernatants remained after depletion of membrane vesicles, with membrane vesicles collected from CPE-treated cells causing only a minor increase in parent cell death.

The nature of the cytotoxic activity in crude supernatants and in membrane vesicle-depleted supernatants was further investigated. Characterization studies determined that this factor appears to be a heat-sensitive protein of 10 to 30 kDa. The addition of 200 μg ml⁻¹ trypsin inhibitor caused a significant reduction in killing of parent cells by membrane vesicle-depleted supernatants. The addition of twice that amount of trypsin inhibitor did not further reduce cytotoxicity (not shown), suggesting that a cytotoxic factor other than the serine protease is also present in supernatants from CPE-treated sensitive cells.

Serine proteases are involved in apoptosis (36), which is consistent with our observation that the development of apoptosis is important for the release of cytotoxic factors into the supernatants of CPE-treated sensitive cells. Whether the serine protease present in supernatants from CPE-treated sensitive cells is directly responsible for parent cell killing or instead activates another factor will require further work to determine. Barely detectable (equivalent to <1 μg/ml of trypsin) protease activity was detected in membrane vesicle-depleted supernatants, and most of this activity was sensitive to trypsin inhibitor (data not shown). Those low levels of serine protease activity in supernatants from CPE-treated cells, coupled with the observation that there appear to be more than one cytotoxic factor in these supernatants, will make future analyses of the cytotoxic factors by conventional biochemical approaches challenging.

The presence of some serine protease activity in supernatants is not surprising given that a number of other host proteins, such as LDH and caspase-3, were also detected in supernatants collected from CPE-treated sensitive cells. However, caspase-3 activity itself was clearly not responsible for the cytotoxic activity in supernatants from CPE-treated sensitive cells. Whether the serine protease present in supernatants from CPE-treated sensitive cells, since inhibition of caspase-3 activity in supernatants from CPE-treated sensitive cells did not reduce the cytotoxic activity of those supernatants.

The discovery in this study that CPE-sensitive cells release a cytotoxic factor(s) to damage other cells that is inherently insensitive to the direct effects of this toxin may have biomedical significance. Since the CPE quantities used in this study have pathophysiologic relevance (37), this bystander killing effect could contribute to CPE-mediated gastrointestinal (GI) disease. In the GI tract, CPE damages sensitive cells after binding to claudin receptors on enterocytes (37). However, the availability of claudin CPE receptors varies considerably among different intestinal segments, along the crypt to villus/surface cell axis in the intestines, by subcellular distribution and with age (38–40). Therefore, during GI disease, it is likely that only some cells respond directly to CPE, as supported by immunolocalization experiments showing CPE binding predominantly to villus tips in rabbit small intestine, where CPE damage begins (37). However, in the GI tract, CPE-
sensitive cells are likely in the proximity of CPE-insensitive cells. Our current work could suggest that CPE induces release of cytotoxic factors from those CPE-sensitive cells, which could damage nearby CPE-insensitive cells and contribute to pathology. In addition, epidemiological evidence and animal model studies strongly suggest that, when preexisting conditions exist that block the development of diarrhea (such as severe constipation or fecal impaction due to medication side effects), CPE can cause enterotoxemia (41, 42). In this situation, the enterotoxin can be absorbed from the intestines into the circulation system, where it then damages organs such as the kidneys and liver. Those organs are also a complex mix of CPE-sensitive and CPE-insensitive cells, where release of cytotoxic factors from CPE-sensitive cells might contribute to pathology by inducing damage to nearby CPE-insensitive cells. These possibilities suggest a need for future studies to examine whether CPE can affect naturally insensitive cells in the intestines or other organs.

Last, there is currently significant interest in translational use of CPE for cancer therapy and diagnosis or drug delivery. It is conceivable that the release of cytotoxic factors from CPE-treated sensitive cells could complicate some of these translational efforts when insensitive cells are in close proximity to CPE-sensitive target cells.

MATERIALS AND METHODS

Materials. Native Clostridium perfringens enterotoxin (CPE) was purified to homogeneity as described previously (43). Rabbit polyclonal antibody was raised against purified CPE as described previously (44). CPE-neutralizing monoclonal antibody 3C9 and nonneutralizing CPE monoclonal antibody 10G6 were prepared previously (45) and purified by standard techniques (45). E. coli transformants producing recombinant CPE (rCPE) or the rCPE-D48A variant (rCPE with D48A change) were enriched to near homogeneity by Talon resin chromatography (Qiagen) as described previously (26). Protein concentrations were determined for differential centrifugation fractions using the Pierce 660 nm protein assay (Thermo Fisher) and for other samples using the Pierce BCA protein assay kit (Thermo Fisher). Protease activity in supernatants was measured using the Pierce protease assay kit (Thermo Fisher).

Treatment of cell cultures with CPE species. Rat1-R12 parental fibroblasts do not naturally express claudin 17, 46, and 47 and are referred to as parent cells. Previously prepared (48) Rat1-R12 fibroblast transfectant cells that stably express claudin-4 are referred to as claudin-4 transfectant cells. Authenticated Caco-2 cells were purchased from ATCC. Unless otherwise specified, cell cultures were treated with Hanks balanced salt solution (HBSS) containing 0.5 or 5 μg ml−1 of a CPE species (CPE, rCPE, or rCPE-D48A) for 1 h at 37°C.

Microscopy. Washed parent cells were suspended at 3 × 105 cells/ml in phosphate-buffered saline (PBS) at room temperature (RT) and then stained with 1 μM CFSE [5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (eBioscience)] for 10 min at RT in the dark. The reaction was stopped by adding 4 to 5 volumes of cold complete minimum essential medium (MEM), followed by incubation on ice for 5 min. After three washes with complete MEM, pure cultures of those stained cells, or unstained claudin-4 transfectant cells, were cultured to confluence in an 8-well microscopy slide (Lab-Tek) coated with Vitrogen plating medium MEM clear (Life Technology), 1 mg ml−1 fibronectin (Fishier), 7.5% bovine serum albumin (BSA) (Life Technology), and 3 mg ml−1 bovine collagen (Fishier). To prepare mixed cultures of unstained claudin-4 transfectant cells together with CFSE-stained parent cells, each cell type was inoculated at a 1:1 ratio and cultured to confluence on a Vitrogen-coated microscopy chamber slide. The pure or mixed cell cultures were treated with CPE, washed twice with PBS, and stained with the fixable viability dye eFluor 450 (eBioscience) at a 1:10,000 dilution for 30 min at 4°C in the dark. After two washes with PBS, the cells were fixed with 4% paraformaldehyde for 20 min in the dark at RT. The fixed cells were washed twice with PBS and then stained with Hoechst 33342 (Invitrogen) at a 1:10,000 dilution for 10 min at RT. Microscopy was performed using an Olympus confocal laser scanning biological microscope (FluoView FV1000) with FV10-ASW (version 1.4) software. All pictures were taken at a magnification of 200×. Quantification of cell viability (eFluor 450-stained cells) or CFSE staining was performed by counting six microscopic fields for each treatment condition in each experiment, with this analysis repeated in three independent experiments.

CPE Western blot analysis of CH-1 formation. CPE-treated Caco-2 cells or claudin-4 transfectant cells were washed with HBSS, suspended in PBS, and centrifuged at 2,000 × g for 3 min. The cell pellet was then resuspended in 50 μl of radioimmunoprecipitation assay (RIPA) buffer and processed for CPE Western immunoblotting as described previously (19). Similarly, supernatants from CPE-treated claudin-4 transfectants or Caco-2 cell cultures were directly analyzed by CPE Western blotting or applied to parent cells for 1 h at 37°C. After the cells were washed, the supernatant-treated parent cells were analyzed by CPE Western blotting (19).

Analysis of culture supernatant effects on cytotoxicity. CPE species-treated confluent parent cells, claudin-4 transfectant cells, or Caco-2 cells were collected by centrifugation at 2,000 × g for 3 min, and supernatants from those centrifugations were applied to fresh confluent parent cell cultures for 1 h at 37°C. Cytotoxicity in these cultures was measured by microscopy (as described above) or by using an MTT assay kit (CellTiter 96 Aqueous one-solution cell proliferation assay; Promega) or LDH release assay (Roche) per the manufacturer’s instructions. LDH release specifically induced by supernatant treatment of parent cells was calculated after subtracting the LDH already present in the supernatants due to its release from CPE-treated Caco-2 cells or claudin-4 transfectants.

Analysis of release of cytotoxic factor(s) by CPE-treated sensitive cells. CPE-treated claudin-4 transfectant cells were centrifuged for 3 min at 2,000 × g. The resultant supernatants were processed to evaluate the protease sensitivity of their cytotoxic activity after treatment with or without 100 μg ml−1 of trypsin (Sigma) for 1 h at 37°C. Trypsin inhibitor (200 or 400 μg ml−1; Sigma) in HBSS was added, and after 30 min at RT, the samples were applied to confluent parent cells for 1 h at 37°C. An MTT assay was then performed to determine cytotoxicity. The size of cytotoxic factor(s) in the supernatants was evaluated by centrifuging the supernatants on 10-kDa- or 30-kDa-cutoff Amicon filters (Millipore). The flow-through and retained fractions were separately applied to confluent cultures of the parent cells, and an MTT assay was performed. The heat sensitivity of cytotoxic factor(s) was evaluated by heating the supernatants at 70°C for 10 min. Heated and unheated supernatants were then separately applied to confluent cultures of the parent cells, and an MTT cytotoxicity assay was performed.

Trypsin sensitivity, trypsin inhibitor sensitivity, heat sensitivity, size of cytotoxic factor(s), and protease activity in supernatants from CPE-treated claudin-4 transfectants that were depleted of membrane vesicles by ultracentrifugation at 100,000 × g for 90 min were examined. An aliquot of those supernatants was also ultrafiltered through 30-kDa-cutoff Amicon filters.

Antibody neutralization of CPE activity. CPE (1 μg ml−1) alone or in the presence of 10 μg ml−1 of MAb3C9 or MAb10G6 was preincubated in HBSS at 37°C for 1 h. For controls, the same amounts of those monoclonal antibodies were similarly preincubated in HBSS without CPE. After preincubation, samples were added to claudin-4 transfectants for 1 h at 37°C. Culture supernatants were then collected by centrifugation at 2,000 × g for 3 min, and supernatants from those centrifugations were applied to parent cells for 1 h at 37°C, followed by cytotoxicity assay using an MTT assay.

Differential centrifugation fractionation of supernatants from CPE-treated sensitive cells. CPE-treated claudin-4 transfectant or Caco-2 cell cultures were centrifuged at 2,000 × g for 3 min, and those super-
natants were subjected to sequential centrifugation (30 min at 10,000 × g to collect large membrane vesicles and then 90 min at 100,000 × g to collect small membrane vesicles [49, 50]). Pelleted membrane vesicles resuspended back to natural (1X) concentrations, or supernatant fractions from centrifugations, were applied to parent cells for MTT cytotoxicity testing or Western blotted with a cadherin antibody (Cell Signaling Technology, Inc.) to validate vesicle depletion from supernatants. For cadherin Western blots, equal amounts of protein were separated by SDS-PAGE on Tris gels (Bio-Rad) and transferred to a polyvinylidene difluoride (PVDF) membrane.

Caspace-3/7 activity in parent cells challenged with supernatants from CPE-treated sensitive cells. Claudin-4 transfectant cells or Caco-2 cells were preincubated for 2 h at 37°C in HBSS with or without 50 μM caspase-3/7 inhibitor N-acetyl-Asp-Glu-Val-Asp-α-(Ac-DEVD-CHO) (Sigma). After two washes with HBSS, 1 μg CPE ml⁻¹ was added in the presence or absence of 50 μM Ac-DEVD-CHO. After 1 h at 37°C, the CPE-treated cells were washed twice with HBSS and collected by centrifugation. The cell pellets were suspended in lysis buffer [50 mM HEPES (pH 7.4), 0.1% 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1 mM dithiothreitol, 10 mM dithiothreitol] and combined in a total 100-μl aliquot of each cell lysate (as prepared above), 10 μl of caspase substrate solution, and assay buffer were combined in a total 100-μl volume and incubated at 37°C for 4 h. Release of p-nitroanilide from the caspase-3 substrate was detected by measuring optical density at 405 nm (OD₄₀₅) using a Bio-Rad plate reader. The amount of substrate cleaved was calculated according to the manufacturer’s instructions. Supernatants from the CPE-treated Caco-2 cells and claudin-4 transfectants were also directly assayed for caspase-3/7 and LDH activity before being applied to parent cells.

Parent cells were also preincubated for 1 h at 37°C in HBSS with a 50 μM concentration of Ac-DEVD-CHO before 1-h challenge with supernatants collected from sensitive cells that had been treated for 1 h with 5 μg ml⁻¹ of CPE. Those supernatant-treated parent cells were assessed for LDH release, and after they were washed, caspase-3/7 activity assay as described above.

Statistical analyses. Multiple comparisons used one-way analysis of variance (ANOVA) with the Dunnett’s multiple comparison test. For pairwise data comparisons, Student’s t test was performed.

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