Using More Than 1 (Path)Way to Kill a Host Cell: Lessons From *Clostridium perfringens* Enterotoxin

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ABSTRACT: *Clostridium perfringens* enterotoxin (CPE) is responsible for the symptoms of common intestinal infections due to *C. perfringens* type F isolates. CPE is a pore-forming toxin that uses certain claudins as a receptor. Previous studies showed that, in enterocyte-like Caco-2 cells, low CPE concentrations cause caspase 3-mediated apoptosis but high CPE concentrations cause necrosis. The recent work published in *mBio* by Shrestha, Mehdizadeh Gohari, and McClane determined that RIP1 and RIP3 are involved in both CPE-mediated apoptosis and necrosis in Caco-2 cells. Furthermore, mixed lineage kinase-domain (MLKL) oligomerization was shown to be important for necrosis caused by CPE, identifying this necrosis as programmed necroptosis. In addition, calpain activation due to Ca^{2+} influx through the CPE pore was identified as a critical intermediate step for MLKL oligomerization and, thus, CPE-induced necroptosis. These findings may have applicability to understand the action of some other pore-forming toxins that induce necroptosis and may also be important for understanding CPE action in vivo.

KEYWORDS: Clostridium perfringens, enterotoxin, pore-forming toxin, necroptosis, calpain

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Introduction

Production of *Clostridium perfringens* enterotoxin (CPE) is essential for *C. perfringens* type F strains to cause intestinal diseases, including food poisoning and nonfoodborne gastrointestinal diseases such as antibiotic-associated diarrhea.[1,2] CPE, a 35-kDa single polypeptide with a unique primary amino acid sequence, belongs to the aerolysin pore-forming toxin family.[1,2] It consists of an C-terminal domain that mediates binding to receptors and an N-terminal domain responsible for oligomerization and pore formation.[1,2]

Early Steps in CPE-Induced Cell Death

As shown in Figure 1, CPE cytotoxicity starts when this toxin binds to receptors, which include certain members of the claudin family of tight junction proteins. This binding results in formation of a small (~90 kDa) complex that contains CPE along with both receptor and nonreceptor claudins. Approximately 6 CPE small complexes then oligomerize into an ~500 kDa prepore located on the host cell plasma membrane surface. A beta hairpin loop then extends from each CPE molecule to create a beta barrel pore that is inserted into the host cell membrane. This CPE pore is permeable to molecules <200 Da, particularly cations.

New Insights Into CPE-Induced Cell Death Pathways

Approximately 15 years ago we reported that, in a concentration-dependent manner, CPE activates 2 different cell death pathways.[3,4] In those studies, low (1 μg/mL) CPE concentrations killed human enterocyte-like Caco-2 cells by a classical caspase-3 mediated apoptosis. However, higher (10 μg/mL) CPE concentrations caused those cells to die from necrosis, although the molecular details of that necrotic process were not defined. Those studies also established that elevated cytoplasmic levels of Ca^{2+} and increased calpain activation are important for the development of either CPE-induced apoptosis or necrosis but, again, mechanistic details were unclear.

Since publication of those initial CPE cell death pathway studies, considerable progress has been achieved in understanding mammalian cell death pathways induced by various other (non-CPE) treatments. In particular, 2 host kinases named receptor interacting serine/threonine kinases 1 and 3 (i.e. RIP1 and RIP3) and a pseudokinase named mixed lineage kinase-domain (MLKL) have been implicated in some cell death pathways.[5] RIP1 and RIP3 help mediate classical caspase-3 mediated apoptosis as part of Complex IIb. However, all three of these proteins are involved in a form of programmed necrosis known as necroptosis. In the necroptotic cell death pathway, RIP1 and RIP3 are located in the necrosome, which promotes oligomerization of MLKL to serve as the executioner for cell death through an incompletely understood process.

Given those advances, our recent *mBio* study used highly specific inhibitors of RIP1 or RIP3 activity, or MLKL oligomerization, to explore if those 3 host proteins play a role in CPE-induced cell death. Results indicated that RIP1 and RIP3, but not MLKL oligomerization, are important for the apoptosis induced when Caco-2 cells are treated with a low CPE concentration. In contrast, RIP1 and RIP3 activity and MLKL oligomerization were all contributors to the necrosis that develops when Caco-2 cells are treated with a high CPE

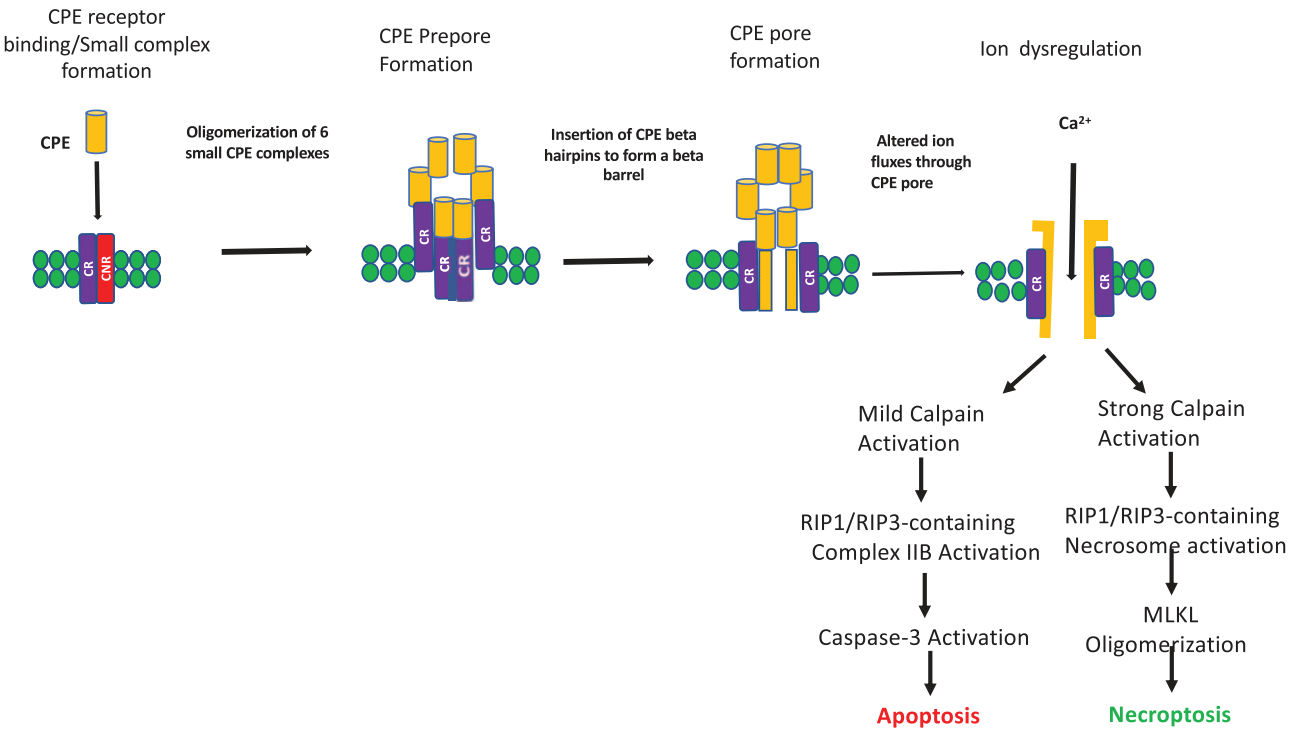

Figure 1. CPE-induced cell death in Caco-2 and T84 cells. CPE (gold) binds to a receptor claudin (purple), to form an ~90-kDa small complex. This small complex also contains nonreceptor claudins (red) which remain associated with bound CPE throughout its action (not shown). Approximately 6 small complexes oligomerize to form a prepore on the host cell surface. Each CPE molecule in the prepore then extends a beta hairpin to create a membrane pore that causes dysregulation of ions (including Ca^{2+} influx). Depending on the CPE concentration applied, there is either limited or strong activation of calpain. With low calpain activation, RIP1 and RIP3, likely acting via complex IIb, cause a caspase-3 activation that then triggers apoptosis. In contrast, strong calpain activation mediates, via the RIP1/RIP3-containing necrosome, MLKL oligomerization to cause necroptosis. See Refs 1, 2, and Shrestha, Mehdizadeh Gohari, and McClane 2019 *mBio* article for more details). CPE indicates *Clostridium perfringens* enterotoxin; MLKL, mixed lineage kinase-domain.

concentration, identifying this cellular necrotic process as necroptosis. In addition, calpain inhibitors were shown to block MLKL oligomerization in Caco-2 cells treated with high CPE concentrations, implicating calpain activation as a key intermediate step in CPE-induced necroptosis. A model incorporating these findings is included in Figure 1. Last, these findings are not specific for Caco-2 cells since similar results were also obtained when T84 human enterocyte-like cells were treated with the same low or high CPE concentrations.

Relevance of These In Vitro Findings for Understanding CPE-Mediated Intestinal Disease

While in vitro results using cultured Caco-2 or T84 cells are interesting, are they germane for understanding CPE-mediated intestinal disease? *C. perfringens* likely produces CPE during sporulation to induce a diarrhea that expels spores from the body and thus propagates disease transmission. In animal models, purified CPE causes severe intestinal damage, including villus shortening, gross epithelial necrosis, and cellular desquamation.[1,6] This intestinal damage is apparently required for the development of diarrhea since intestinal fluid loss in CPE-treated animal models (1) correlates with the onset of intestinal damage and (2) can only be produced using concentrations of purified CPE that are sufficient to cause intestinal damage.[1,6]

The CPE concentrations used in our Caco-2 and T84 cell studies have pathophysiologic relevance as they overlap CPE levels measured in diarrheic feces from patients with CPE-mediated intestinal disease, which range from <1 to >100 μg/mL of the enterotoxin.[7] The broad range of CPE concentrations present in diarrheic feces supports the concept that it may be beneficial if, during disease, CPE broadly induces intestinal cell death and consequent tissue damage at both low and high concentrations, consistent with our cell culture results. Interestingly, ⩾ 10 μg/mL of purified CPE is needed to cause intestinal pathology in rabbit small intestinal loop models,[6] which is higher than the CPE concentrations measured in diarrheic feces of some patients. This apparent paradox could be explainable by (1) differences in CPE sensitivity between animal models vs humans, (2) lowering of fecal CPE levels due to degradation by fecal proteases, and/or (3) late collection of some diarrheic fecal samples, ie, collection after CPE stool levels had already peaked. Another possible explanation could be that a *C. perfringens* factor(s) not present in purified CPE preparations significantly enhances the activity of lower CPE concentrations during intestinal infection. This hypothesis is supported by some in vitro data, ie, CPE-producing type F strains often make NanI sialidase, which increases CPE binding and cytotoxicity for Caco-2 cells by ~2- to 3-fold.[8]

To date, there has been only limited direct study of CPE-induced cell death pathway activation in the intestines. A recent study[9] demonstrated some caspase-3 activation in

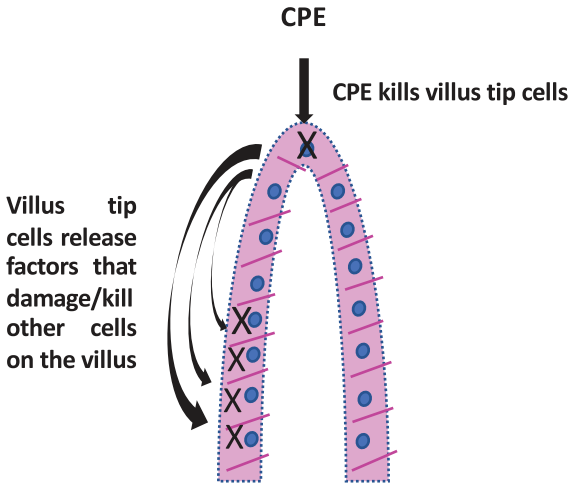

Figure 2. Model for CPE-induced in vivo cell death and intestinal damage. CPE binds to claudin receptors on villus tip cells. Those cells die and release factors, possibly a serine protease, that cause a bystander killing effect on naturally CPE-insensitive intestinal cells located further down the villus. Based on research described in Shrestha et al.[11] CPE indicates *Clostridium perfringens* enterotoxin.

CPE-treated rabbit small intestine. However, this activation initiated after the onset of intestinal damage. Furthermore, caspase-3 inhibitors were unable to block the development of CPE intestinal damage. Whether CPE causes intestinal necroptosis has not yet been examined.

There is another interesting, yet complicating, aspect of CPE intestinal action. This enterotoxin binds primarily to villus tip cells yet damages the entire villus.[10] That observation prompted recent in vitro studies[11] showing CPE-induced death of sensitive Caco-2 cells causes release of factors, possibly including a serine protease, that kill CPE-insensitive cells. Extrapolating this observation to the intestines may suggest that CPE kills sensitive villus tip cells, which then release factors to cause bystander cell killing of insensitive cells further down the villus. If correct, the bystander killing effect may actually be responsible, via a still unknown cell death pathway, for much of the intestinal damage observed in CPE-treated intestines. A proposed model incorporating the involvement of bystander killing in CPE in vivo effects is shown in Figure 2.

Possible Pertinence of These New CPE Findings for Understanding Cell Death Mechanisms of Other Pore-Forming Toxins (PFTs)

PFTs such as CPE represent the largest single class of bacterial toxins. Studies prior to our recent *mBio* CPE paper had already demonstrated that several other PFTs can induce necroptosis through processes that apparently involve cytoplasmic ion dysregulation.[12-14] However, the mechanisms behind those processes have been unclear. For example, the cause of the cytoplasmic ion change in necroptotic cells had not been clearly determined. Some studies with other (non-PFT) factors had reported that oligomerization of MLKL during necroptosis can itself affect intracellular ion levels.[15] Alternatively, ion dysregulation caused directly by PFT pores might promote MLKL oligomerization and thus necroptotic cell death. Also, it had been unclear whether intermediate host factors beyond the necrosome can be involved in PFT-induced MLKL oligomerization.

Given those uncertainties, our recent *mBio* paper offers 2 potentially more generalizable contributions for understanding necroptosis induced by at least some other PFTs. First, these CPE results demonstrate that Ca^{2+} influx through a PFT pore to activate calpain can mediate MLKL oligomerization and necroptosis. It remains possible that MLKL oligomerization then causes some secondary ion dysregulation to further potentiate CPE-induced cell death. Second, this CPE research establishes the principle that, beyond the necrosome, cellular intermediates like activated calpain can also help mediate MLKL oligomerization and PFT-induced necroptosis. It is possible that some PFTs, particularly those causing Ca^{2+} influx, also use a calpain-mediated pathway to cause necroptosis.

Unresolved Questions for Future Research

The above discussion raises a number of interesting and important questions regarding CPE action and, more generally, PFT-induced cell death pathways. Some topics for future research include: Does CPE-induced bystander killing occur in the intestines? Does CPE directly, or via bystander killing, cause necroptosis in the intestines? If so, is this effect important for causing intestinal tissue damage? What is the target of activated calpain during apoptosis or necroptosis in CPE-treated cells? How does this lead to MLKL oligomerization in cells treated with high CPE doses? Regarding other PFTs, do some of those resemble CPE by causing (in a dose-dependent nature) apoptosis and necroptosis? Is calpain activation an important intermediate when other PFTs induce necroptosis?

Author Contributions

AS and BMC both wrote the text and prepared the figures together.

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