Enteropathogenic *E. coli*-induced barrier function alteration is not a consequence of host cell apoptosis

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Enteropathogenic *E. coli*-induced barrier function alteration is not a consequence of host cell apoptosis. Am J Physiol Gastrointest Liver Physiol 294: G1165–G1170, 2008. First published March 20, 2008; doi:10.1152/ajpgi.00596.2007.—Enteropathogenic *Escherichia coli* (EPEC) is a diarrheagenic pathogen that perturbs intestinal epithelial function. Many of the alterations in the host cells are mediated by effector molecules that are secreted directly into epithelial cells by the EPEC type III secretion system. The secreted effector molecule EspF plays a key role in redistributing tight junction proteins and altering epithelial barrier function. EspF has also been shown to localize to mitochondria and trigger membrane depolarization and eventual host cell death. The relationship, if any, between EspF-induced host cell death and epithelial barrier disruption is presently not known. Site-directed mutation of leucine 16 (L16E) of EspF impairs both mitochondrial localization and consequent host cell death. Although the mutation lies within a region critical for type III secretion, EspF(L16E) is secreted efficiently from EPEC. Despite its inability to promote cell death, EspF(L16E) was not impaired for tight junction alteration or barrier disruption. Consistent with this, the pan-caspase inhibitor Q-VD-OPH, despite reducing EPEC-induced host cell death, had no effect on infection-mediated barrier function alteration. Thus EPEC alters the epithelial barrier independent of its ability to induce host cell death.

**tight junction; pathogenesis; EspF**

**ENTEROPATHOGENIC ESCHERICHIA COLI (E. coli) (EPEC)** is a gram-negative extracellular intestinal pathogen that causes diarrhea (20). Children in developing countries are particularly susceptible to this infection (12). The EPEC type III secretion system (T3SS), an elaborate multi-protein structure, conveys specific bacterial effector proteins directly into host intestinal epithelial cells. These effector molecules manipulate host cell physiology, presumably to facilitate bacterial colonization and dispersal. For instance, EPEC modulates the survival of host epithelial cells by interfering with pro- and antiapoptotic signals (12). The net effect is a weak induction of cell death, displaying features of both apoptosis and necrosis, following in vitro infection of epithelial cells (8).

The intestinal epithelium is a single layer of cells whose apical junctional regions are comprised of multimolecular complexes known as tight junctions (TJs) (30). TJs form a regulable barrier to the paracellular transport of water, ions, and immune cells. EPEC redistributes the TJ proteins including occludin and zonula occludens-1, and thereby disrupts the barrier function of epithelial monolayers. Various studies have demonstrated the disruption of epithelial barrier function as a result of apoptosis induced by chemicals, immune molecules, and pathogen-associated factors (1, 6, 28).

The relationship between EPEC-induced host cell death and epithelial barrier disruption is presently unknown. One of the type III-secreted effector molecules, EspF, has been reported to localize to the host cell mitochondria and promote cell death (9, 19, 21). Site-directed mutagenesis of a single amino acid in the mitochondrial localization signal of EspF, leucine 16 (L16E), prevented its movement to the mitochondria and curtailed EPEC-induced apoptosis of epithelial cells (9, 17). Interestingly, EspF also plays a key role in the disruption of epithelial barrier function by redistributing TJ proteins (17). Thus EPEC induces barrier disruption and apoptosis (19).

EspF is a 206-amino-acid proline-rich protein that interacts with multiple host proteins including cytokeratin 18, Abcf2, sorting nexin 9 and N-WASP (2, 16, 23, 33). EspF could impact host cell survival and epithelial barrier function by related or independent pathways. To determine the relationship between EspF-induced host cell death and its effect on barrier function, we complemented the ΔespF strain UMD874 with a plasmid encoding either wild-type (WT) EspF or EspF(L16E) and explored the effect of these strains on host cell function. Furthermore, the effect of a pharmacological inhibitor of apoptosis on EPEC-induced barrier function alteration and host cell death was determined. Our data suggest that apoptosis does not significantly contribute to EPEC-induced barrier disruption.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** WT *E. coli* O127:H6 strain E2348/69 and the ΔespF derivative UMD874 have been described previously (17). EspF was amplified by using primers CCATGGTTAATGGAATTAGTAA and YTTCGATWGYTCATAGGCAGC, and the largest (full-length) fragment was cloned into pTrHis2-TOPO (Invitrogen, Carlsbad, CA) in-frame with the COOH-terminal Myc and His tags to generate pRPK14. Site-directed mutagenesis was performed using the Quickchange II kit (Stratagene, La Jolla, CA) to alter codon 16 (to encode glutamic acid instead of leucine) in the espF reading frame in pRPK14 to create pAW1. The mutation in UMD874 was complemented with pRPK14 (encoding WT EspF) pAW1 [encoding EspF(L16E)] to generate strains GH290 and GH406, respectively.

**Western Blot analysis.** EspF expression and secretion was monitored as described previously (32). Briefly, UMD874 and GH406 strains were subcultured in DMEM (described below) to midlog phase,
and the bacteria were harvested by centrifugation. Bacterial pellets and TCA-precipitated supernatants were resuspended in Laemmli loading buffer and electrophoresed on a 6% SDS-polyacrylamide gel using the Protean II Xl apparatus (Bio-Rad, Richmond, CA). The separated proteins were transferred to nitrocellulose membranes (Transblot Cell Apparatus, Bio-Rad), blocked with 5% nonfat milk in Tris-buffered saline containing Tween 20 (TBST), and then incubated with antibodies to EspF (32). Following the required washes, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody and subsequently developed with the enhanced chemiluminescence (ECL) Western blotting substrate for HRP (Amersham Biosciences, Piscataway, NJ).

Cell lines and infection. SGLT-expressing Caco-2 BBE (C2BBE) intestinal epithelial cells (24, 31) were cultured in 25 mM glucose DMEM, 10% fetal bovine serum, 20 mM HEPEs, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in the presence of 5% CO2. T84 epithelial cells were grown in a 1:1 (vol/vol) mixture of DMEM and Ham’s F-12 supplemented with 6% newborn calf serum. Our observations were similar in Caco-2 and T84 cells, and data for key experiments are provided from both cell lines. Although both cell lines respond similarly to EPEC infection with regards to the cell death and barrier disruption phenotypes, T84 cells are relatively refractory and display the phenotypes at later time points, or in the presence of a higher inoculum of EPEC. For barrier function studies, epithelial cell monolayers were grown on permeable supports (Costar Transwells). We utilized an established model for infecting epithelial cells (29, 33). Briefly, Luria-Bertani broth-grown EPEC strains were subcultured in antibiotic-free DMEM and cultured to midlog growth phase. EspF expression in the complemented strains was induced by the addition of 50 μM isopropyl β-D-1-thiogalactopyranoside (IPTG); IPTG was maintained during the course of the infection.1 Epithelial monolayers were infected at an approximate multiplicity of infection (MOI) of 1:100 and incubated in antibiotic- and serum-free medium at 37°C in a 5% CO2 water-jacketed incubator for the indicated time periods.

Cell death assays and immunofluorescence microscopy. Host cell death was evaluated by the ethidium homodimer uptake method using the Live/Dead viability/cytotoxicity kit (Invitrogen) per the manufacturer’s instructions. For quantitation, cells staining red were counted as dead (32). Cell death was evaluated by the ethidium homodimer uptake method using the Live/Dead viability/cytotoxicity kit (Invitrogen) per the manufacturer’s instructions. For quantitation, cells staining red were counted as dead (32). Cell death was evaluated by the ethidium homodimer uptake method using the Live/Dead viability/cytotoxicity kit (Invitrogen) per the manufacturer’s instructions. For quantitation, cells staining red were counted as dead (32). Cell death was evaluated by the ethidium homodimer uptake method using the Live/Dead viability/cytotoxicity kit (Invitrogen) per the manufacturer’s instructions. For quantitation, cells staining red were counted as dead (32).

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RESULTS

EsPF-L16E is not impaired for secretion. The type III secretion signal for EspF, localized to the first twenty amino acids of the protein, overlaps with the mitochondrial targeting sequence (5, 19). The site-directed alteration of L16E could, therefore, interfere with the expression and/or secretion of EspF. To evaluate this, protein extracts and TCA-precipitated supernatants of the pEspF- and pEspF(L16E)-complemented ΔespF strains, respectively, were examined by Western blot analysis. Plasmid-encoded EspF and EspF(L16E) were both expressed and secreted in an IPTG-inducible manner, and the mutant protein was secreted in amounts comparable to WT EPEC (Fig. 1). Both the plasmid-encoded proteins include COOH-terminal Myc and His tags. The additional faster migrating bands result from NH2-terminal cleavage of EspF and have been reported earlier by Nougayrede and Donnenberg (21).

EspF(L16E) is impaired for inducing host cell apoptosis. Using site-directed mutagenesis, Nagai et al. (19) demonstrated that the 16th leucine of EspF was critical for mitochondrial localization and consequent induction of apoptosis in HeLa cells. To confirm this under the conditions of infection and expression in our Caco-2 system, epithelial cells infected with various EPEC strains were examined for apoptosis using the ethidium homodimer uptake assay. Dead cells are marked by red fluorescence since their compromised membranes are permeable to this high affinity nucleic acid stain. Relative to WT EPEC, ΔespF was significantly impaired for inducing host cell death (8.37 ± 1.43% and 4.30 ± 0.61% dead epithelial cells per field following infection with EPEC vs. ΔespF, respectively; Fig. 2, A and B) (9, 19). The ΔespF defect in cytotoxicity was complemented by plasmids encoding WT but not EspF(L16E) [11.07 ± 1.79% vs. 4.83 ± 0.72% dead host cells following infection with ΔespF complemented with plasmid-encoded WT EspF vs. EspF(L16E)]. Typically, T84 epithelial cells are relatively more resilient to the effects of EPEC, and a higher MOI (~200) of the bacteria is used for infections. As with Caco-2 cells, however, EPEC induced apoptosis of T84 cells in an EspF-dependent fashion. Plasmid-encoded EspF(L16E) failed to complement ΔespF for inducing host cell death (data not shown).

EspF-induced redistribution of occludin is not dependent on its proapoptotic activities. To explore the relationship between EspF-induced cell death and TJ disruption, Caco-2 and T84 intestinal epithelial monolayers were infected with WT, ΔespF, ΔespF/pEspF, and ΔespF/pEspF(L16E). As reported earlier, occludin is distributed in a uniform chicken-wire pattern at the

![Image](http://ajpgi.physiology.org/)

Fig. 1. Alteration of Leucine 16 (L16E) does not impair EspF secretion. Bacterial pellets and TCA-precipitated supernatants from DMEM-grown ΔespF, ΔespF/pEspF, and ΔespF/pEspF(L16E) were separated by SDS-PAGE and immunoblotted for EspF. The COOH-terminal Myc/His-tagged EspF and EspF(L16E) are indicated by an arrowhead. The faster migrating bands represent cleaved EspF (21). IPTG, isopropylthiogalactoside.)
edge of epithelial cells, and these “strands” of occludin are disrupted in EPEC-infected monolayers (arrowheads in Fig. 3) (18, 29). Minimal occludin redistribution, if any, was observed following infection with \( \nabla H9004 \) espF (Fig. 3) (29). Plasmids encoding either WT EspF or EspF(L16E) efficiently complemented this \( \nabla H9004 \) espF defect, suggesting that EspF localization to the mitochondria, or subsequent host cell death, is not required for its ability to mediate TJ alterations.

EspF-induced barrier function alteration is independent of its effects on host cell death. To explore the role of EspF-dependent apoptosis in barrier disruption, Caco-2 cells grown on permeable filters were infected with WT, \( \Delta espF \), \( \Delta espF/pEspF \), and \( \Delta espF/pEspF(L16E) \). TER, a measurement of paracellular permeability, was monitored over time. As reported previously, a progressive decrease in TER, reflective of a loss in barrier function, was observed in monolayers infected with...
WT EPEC but not ΔespF (17). Complementation with plasmid-encoded EspF restored the phenotype (Fig. 4) (32). Interestingly, plasmid-encoded EspF(L16E) was equally efficient at complementing this phenotype, suggesting that EspF disrupts the epithelial barrier disruption phenotype independent of its mitochondrial localization or its ability to cause host cell death. Similar observations were made using T84 epithelial monolayers (data not shown).

The pan-caspase inhibitor Q-VD-OPH curtails EPEC-induced death of intestinal epithelial cells. EPEC, in part via EspF, stimulates the activation of caspases, eventually leading to host cell death. To establish the role of caspases in infected cells, the effect of the pan-caspase inhibitor Q-VD-OPH on EPEC-induced host cell death was evaluated. T84 epithelial monolayers were incubated with 40 μM Q-VD-OPH for 1 h and then infected with EPEC with continued maintenance of the inhibitor. Although dead cells were observed in EPEC-infected T84 monolayers, they were fewer in number compared with similarly infected Caco-2 monolayers (Fig. 5, com-
pare with Fig. 2). Infection-induced host cell death was almost completely reversed in the presence of the pan-caspase inhibitor Q-VD-OPH.

Inhibition of caspases does not have an impact on EPEC-induced barrier disruption. To independently assess whether apoptosis contributes to EPEC-induced barrier function alteration, the effect of the pan-caspase inhibitor Q-VD-OPH on TER was explored. T84 monolayers were pretreated with 50 μM Q-VD-OPH for 1 h; subsequently, the monolayers were infected with EPEC for an additional 4 h in the continued presence of the inhibitor. Measurement of TER of infected T84 monolayers revealed that Q-VD-OPH did not significantly affect EPEC-induced barrier disruption (Fig. 6). Consistent with the EspF(L16E) data above, these studies exclude a causal relationship between apoptosis and epithelial barrier function alteration following EPEC infection. Similarly, Q-VD-OPH had no effect on EPEC-induced TER reduction in Caco-2 cells (data not shown).

DISCUSSION

Although several groups have explored the effect of EPEC on host cell survival, the role of apoptosis in the pathogenesis of this organism is not entirely clear. There is considerable evidence that the secreted protein EspF localizes to the mitochondria of EPEC-infected cells and promotes permeabilization of the mitochondrial membrane, release of cytochrome c, and cleavage of caspase-3, implicating a role for this protein in host cell death (19, 21). Even so, EPEC itself is a relatively weak inducer of apoptosis. This could be because EPEC stimulates the EGF receptor, NF-κB, and PKC pathways, all of which promote survival (10, 26, 27). Moreover, in a rabbit model of EPEC infection, apoptosis was unchanged or possibly less than baseline levels (14).

Since EPEC EspF is substantially responsible for infection-induced disruption of epithelial TJs and consequent loss of barrier function, we investigated whether the effects of EspF on host cell death had a role in alteration of paracellular permeability. Two independent lines of investigation failed to demonstrate a causal link between apoptosis and TJ disruption in infected monolayers. First, a site-directed mutant of EspF impaired for causing host cell death promoted TJ disruption and altered barrier function. Second, the pan-caspase inhibitor Q-VD-OPH did not interfere with EPEC-induced barrier disruption.

These results are consistent with what is presently known about these two phenotypes in infected intestinal epithelial cells. First, EPEC-induced effects on tight junctions are observed as early as 2 h postinfection, at a point of time when very little host cell death is evident (18). Second, one of the limitations of studies demonstrating mitochondrial targeting and apoptosis related to EPEC infection is the need to utilize a high MOI to see such effects (21, 22) (data not shown). Third, it has been suggested that the EPEC mitochondria-associated protein (Map) and EspF work in concert to effect changes in TJs in a manner that is independent of Map mitochondrial targeting (11). Finally, there is some evidence that the COOH-terminal region of EspF, lacking the mitochondrial localization signals, can disrupt the epithelial barrier (25). In this context, our findings suggest that EspF features independent of its mitochondrial localization, such as the interaction of its COOH-terminal domains with N-WASP, contributes to epithelial barrier function disruption. Although our studies rule out apoptosis as a primary cause for EPEC-mediated TJ alterations, we cannot preclude the possibility that cell death may augment barrier disruption at later points of infection. Also, apoptosis may contribute to small conductive leaks, as has been observed in response to TNF-α-induced single cell apoptotic events (13).

Apoptosis-dependent barrier function alterations, however, have been observed in other systems. TNF-α-treated HT-29/B6 cells display a progressive decrease in TER, which was attenuated by the caspase inhibitor, Z-VAD-FMK (3). Caspase-dependent cleavage of occludin was considered to be at least partially responsible for this phenotype. Agonists of the G protein-coupled receptor proteinase-activated receptor (PAR1) similarly induced apoptosis and concomitantly increased epithelial permeability in a caspase-3-dependent manner in the nontumorigenic SCBN cell line, as well as in mouse colonic epithelium (7). Caspase-3 inhibitors, as well as myosin light chain kinase inhibitors, abolished PAR1-dependent TJ alterations. Some strains of the intestinal pathogen Giardia lamblia (G. lamblia) induce apoptosis, which in turn results in increased paracellular permeability (6). Pharmacological inhibition of caspase-3 blocked G. lamblia-induced barrier disruption, suggesting a causal role for epithelial apoptosis in barrier disruption. Similarly, it has been shown that single-cell apoptosis in TNF-α-treated monolayers leads to transient small conductive leaks (13). On the other hand, the pan-caspase inhibitor Z-VAD-FMK failed to block cytokine (IFN-γ and TNF-α)-mediated epithelial barrier function alteration (4), possibly suggesting the existence of compensatory mechanisms that maintain monolayer integrity.

Studies from various laboratories suggest a complex multifunctional role for EspF. Aside from its role in apoptosis and barrier disruption, EspF inhibits phosphatidylinositol-3-kinase-dependent uptake of EPEC into macrophages by a process independent of its localization to the mitochondria (25). The ability of this protein to orchestrate such diverse host cell functions and the role of these changes in causing disease remain to be understood.

NOTE ADDED IN PROOF

Since the submission of this manuscript, a study examining the effect of caspase inhibitors on EPEC-induced epithelial barrier disruption independently arrived at the same conclusions. Flynn AN, Buret AG. Caspases-3, -8, and -9 are required for induction of epithelial cell apoptosis by enteropathogenic E. coli but are dispensable for increased paracellular permeability. Microb Pathog 44: 311–319, 2008.

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REFERENCES


