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BRIEF COMMUNICATION

# *Bacteroides fragilis* Toxin Induces IL-8 Secretion in HT29/C1 Cells through Disruption of E-cadherin Junctions

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Enterotoxigenic *Bacteroides fragilis* (ETBF) is a human gut commensal bacteria that causes inflammatory diarrhea and colitis. ETBF also promotes colorectal tumorigenesis in the Min mouse model. The key virulence factor is a secreted metalloprotease called *B. fragilis* toxin (BFT). BFT induces E-cadherin cleavage, cell rounding, activation of the β-catenin pathway and secretion of IL-8 in colonic epithelial cells. However, the precise mechanism by which these processes occur and how these processes are interrelated is still unclear. E-cadherin form homophilic interactions which tethers adjacent cells. Loss of E-cadherin results in detachment of adjacent cells. Prior studies have suggested that BFT induces IL-8 expression by inducing E-cadherin cleavage; cells that do not express E-cadherin do not secrete IL-8 in response to BFT. In the current study, we found that HT29/C1cells treated with dilute trypsin solution induced E-cadherin degradation and IL-8 secretion, consistent with the hypothesis that E-cadherin cleavage causes IL-8 secretion. However, physical damage to the cell monolayer did not induce IL-8 secretion. We also show that EDTA-mediated disruption of E-cadherin interactions without E-cadherin degradation was sufficient to induce IL-8 secretion. Finally, we determined that HT29/C1 cells treated with LiCl (β-catenin activator) induced IL-8 secretion in a dose-dependent and time-dependent manner. Taken together, our results suggest that BFT induced IL-8 secretion may occur by the following process: E-cadherin cleavage, disruption of cellular interactions, activation of the β-catenin pathway and IL-8 expression. However, we further propose that E-cadherin cleavage per se may not be required for BFT induced IL-8 secretion.

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## INTRODUCTION

Enterotoxigenic *Bacteroides fragilis* (ETBF) is an intestinal bacteria that has been associated with inflammatory bowel disease and colorectal cancer in humans (1,2). ETBF also cause diarrhea and colitis in both livestock and laboratory animals (3-7). In the Min mouse model, ETBF promotes colonic tumorigenesis via the Th17/IL-23 pathway (8). The only known virulence factor specific for ETBF is the secreted 20 kDa metalloprotease called *B. fragilis* toxin (BFT) (9,10). Addition of purified BFT to colonic epithelial cell lines induces several distinct changes. These include ectodomain cleavage of E-cadherin, morphological "rounding" of cells and secretion of IL-8 (11-14). E-cadherin is a 120 kDa type I transmembrane protein essential to the formation of intercellular adhesion of adjacent epithelial cells (15). The cytoplasmic domain of E-cadherin is bound to β-catenin, which in turn associates with α-catenin and cytoskeletal actin (16).

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Abbreviations: ETBF, enterotoxigenic *Bacteroides fragilis*; BFT, *Bacteroides fragilis* toxin; EDTA, ethylenediaminetetraacetic acid; LiCl, lithium chloride; IL-8, interleukin-8

These associations result in formation of a stable epithelial monolayer which provides a protection barrier against infiltration of external insults. The loss of this epithelial integrity results in inflammatory disorders including colitis. BFT induces rapid cleavage of the extracellular domain of E-cadherin which result in cell rounding and loss of epithelial integrity. Subsequent E-cadherin degradation by $\gamma$-secretase releases the bound $\beta$-catenin and nuclear translocation of $\beta$-catenin activates the $\beta$-catenin-TCF-dependent pathway (17).

To date, several cytokines and chemokines have been identified to be secreted in response to BFT treatment of intestinal epithelial cells: TGF-$\beta$, ENA-78, GRO-$\alpha$, MCP-1 and IL-8 (13,14,18). IL-8 is a potent inflammatory chemokine that is quickly secreted in response to microbial insults and functions to recruit neutrophils to sites of damage. IL-8 induction can occur through activation of the NF-$\kappa$B and MAPK pathways. Using hepatoma cells, Levy et al. found that stimulation of the $\beta$-catenin pathway induces expression of IL-8 due to the presence of a unique consensus Tcf/Lef site that is critical for IL-8 activation by $\beta$-catenin (19). Taken together, we propose a model for BFT-induced IL-8 secretion in which the enzymatically active BFT induces E-cadherin degradation, which results in release of the bound $\beta$-catenin that in turn translocate into the nucleus and actives IL-8 expression. In this study, we present data suggesting that activation of the $\beta$-catenin pathway in the colonic epithelial cell line by disruption of the E-cadherin junction is sufficient to induce IL-8 secretion.

## MATERIALS AND METHODS

### Cell culture and reagents

The human colonic epithelial cells line (HT29/C1) was originally obtained from Dr. Daniel Louvard, Institut Pasteur, Paris, France). HEK293/17 cells were purchased from ATCC. Cells were cultured in 10% FBS-DMEM containing gentamicin (100 ug/ml) and penicillin/streptomycin. All cell culture reagents were purchased from GIBCO BRL Life Technologies (Rockville, MD, USA). Cells were grown to subconfluent monolayers (~70%) in 6-well plates. The cells were then washed with serum-free DMEM three times and then cultured with 3 ml of serum-free DMEM containing purified BFT (100 ng/ml), EDTA (Sigma-Aldrich, USA), NaCl (Sigma-Aldrich, USA), LiCl (Sigma-Aldrich, USA), DSS (MW 30,000~45,000; MP Biochemicals, USA), 0.05% trypsin solution (Gibco, USA) or recombinant human IL-1$\beta$ (50 ng/ml)(R&D Systems, USA) for 24 hr. To induce physical damage to cells, the subconfluent monolayer was scratched full length with a 1 ml pipette tip either 5 or 20 times.

### IL-8 ELISA and Western blot analysis

For IL-8 ELISA, cell culture supernatants were harvested, clarified by centrifugation and stored at $-20^{\circ}$C until analyzed by an IL-8 ELISA kit (R&D Systems, USA). Western blot analysis was performed as previously described (4). In brief, cells were washed two times with ice-cold PBS and lysed on ice for 15 min in RIPA buffer (Sigma-Aldrich, USA) containing protease inhibitors (Roche, USA). Clarified lysates were stored at $-20^{\circ}$C until analyzed by Western blot analysis using monoclonal antibodies against the extracellular domain of E-cadherin (H108, Santa Cruz Biotechnology, USA) or $\beta$-actin (Sigma-Aldrich, USA). Lysates were electrophoresed under reducing conditions on 12% SDS-PAGE gels and then transferred to 0.22-$\mu$m nitrocellulose membranes. Appropriate HRP-conjugated secondary antibodies and SuperSignal West Pico chemiluminescent substrates (Pierce, USA) were used to develop the bands on X-ray film.

### Data analysis

Statistical analysis and graphic presentation were performed using GraphPad Prism 5. p-values were calculated using Anova. Values are shown as mean and standard error of mean (SEM). Data were collected from 3~5 independent experiments. A p-value$<$0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

As previously reported, BFT induces IL-8 secretion in the colonic epithelial cell line HT29/C1 which expresses E-cadherin (Fig. 1A). The induction of IL-8 secretion is considered to be due to cleavage of E-cadherin which releases the intracellular bound $\beta$-catenin leading to IL-8 secretion. Cells that do not express E-cadherin such as HEK293/17 cells do not secrete IL-8 in response to BFT treatment although these cells do have the capacity to release potent levels of IL-8 in response to inflammatory cytokine stimulation such as IL-$\beta$ (Fig. 1A). These results as well as results from other studies suggest that induction of IL-8 by BFT requires E-cadherin cleavage (4). If induction of IL-8 is due to disruption of the E-cadherin junction by BFT mediated cleavage of E-cadherin, then enzymatic cleavage of the E-cadherin ectodomain by other en-

zymes should also induce IL-8. To test this hypothesis, HT29/C1 cells were treated with dilute trypsin for 24 hr and the supernatant assessed for IL-8 secretion by ELISA. We found that trypsin treated HT29/C1 cells showed a dramatic loss of the full length 120 kDa E-cadherin protein and become refractile indicating a loss of cell-to-cell contact (data not shown). In trypsin treated cells, IL-8 secretion was induced in a trypsin concentration dependent manner suggesting that E-cadherin cleavage resulted in IL-8 induction (Fig. 1B). Physical damage of the cellular monolayer was insufficient to induce IL-8 secretion as scratching the subconfluent monolayers with a sterile pipette tip either 5 times or 20 times did not induce IL-8 secretion (Fig. 1B).

The results from the BFT treatment and trypsin treatment strongly suggest that cleavage of E-cadherin is the underlying reason for IL-8 induction. Homophilic E-cadherin interactions between adjacent epithelial cells require the presence of calcium to mediate a stable interaction. Addition of the calcium-chelating EDTA is known to disrupt this interaction without inducing E-cadherin cleavage. We examined if disruption of the E-cadherin homophilic interaction was sufficient to induce IL-8 addition. HT29/C1 cells were treated with different doses of EDTA (0, 1, 2.5, 5 mM) for 24 hr and the secreted IL-8 examined by ELISA. We found an EDTA dose-dependent increase in IL-8 secretion (Fig. 2A). The addition of EDTA did not change any noticeable changes in protein levels of the full length E-cadherin and no evidence of E-cadherin cleavage as determined by Western blot analysis (Fig. 2B). Similarly, we found that physical damage of the HT29/C1 cell monolayer using a pipette tip did not induce E-cadherin cleavage (Fig. 2B). These results suggest that disruption of E-cadherin interaction without E-cadherin cleavage is sufficient to induce IL-8 secretion.

The promoter of the IL-8 gene contains a binding site for the β-catenin/TCF complex (19). This observation suggests that activation of the β-catenin pathway may be sufficient to induce IL-8 secretion in HT29/C1 cells. To determine if HT29/C1 cells can secrete IL-8 through activation of the β-catenin pathway, we treated HT29/C1 cells with LiCl which is known to stimulate the β-catenin pathway (19,20). HT29/C1 cells were treated with different concentrations of LiCl for 24 hr and IL-8 secretion determined by ELISA. We found a LiCl dose-dependent increase in IL-8 secretion beginning at 10 mM which then peaked at 50 mM. At 100 mM LiCl, IL-8 secreted was completely inhibited to background levels suggesting an optimal dose of 50 mM (Fig. 3A). Equal concentrations of NaCl served as a control for LiCl treatment which did not induce IL-8 secretion at all concentrations used (Fig. 3A). Next, we conducted a time course experiment with LiCl

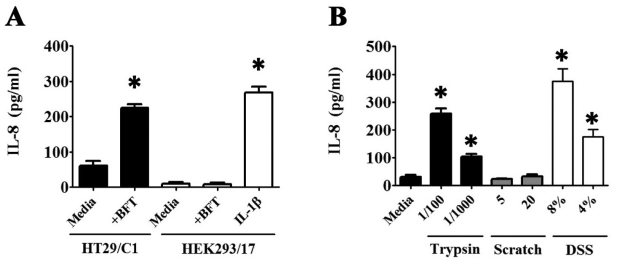


**Figure 1.** Induction of IL-8 secretion by various stimuli. (A) HT29/C1 cells or HEK293/17 cells were cultured with BFT (100 ng/ml) or IL-1β (50 ng/ml) for 24 hr and IL-8 secretion assessed by ELISA. (B) HT29/C1 cells were cultured with diluted trypsin solution (1/100, 1/1,000) or dextran sulfate sodium (DSS) for 24 hr and IL-8 secretion assessed by ELISA. To induce physical damage, the subconfluent cell monolayer was scratched in a line with a pipette tip either 5 or 20 times. Asterisks indicate $p<0.05$. Data are shown as mean and SEM.

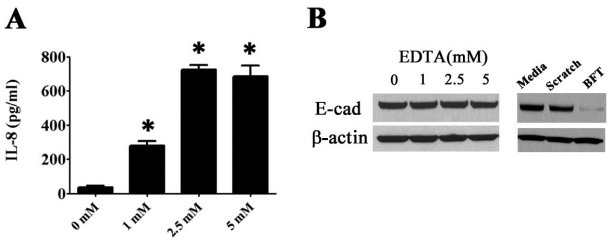


**Figure 2.** Disruption of E-cadherin interaction but not physical damage induces IL-8 secretion. Subconfluent HT29/C1 cells were cultured with EDTA, scratched (20 times) or treated with purified BFT (100 ng/ml) for 24 hr. (A) IL-8 secretion. (B) Western blot analysis of E-cadherin and β-actin. Cells were treated with Asterisks indicate $p<0.05$. Data are shown as mean and SEM.

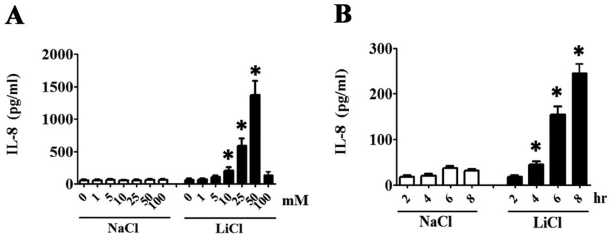


**Figure 3.** LiCl treatment induces IL-8 secretion in HT29/C1 cells. Cells were cultured with (A) different concentrations of LiCl for 24 hr or (B) different lengths of time with 50 mM of LiCl. IL-8 secretion was assessed by ELISA. Asterisks indicate $p<0.05$. Data are shown as mean and SEM.

by treating HT29/C1 cells with 50 mM LiCl for 2, 4, 6 or 8 hrs and measured IL-8 by ELISA. We found an increase in IL-8 secretion beginning at 2 hr post-treatment and thereafter gradually increased over time (Fig. 3B). These results suggest that activation of the β-catenin pathway is sufficient to induce IL-8 secretion in the HT29/C1 cell line.

ETBF has been suggested to contribute to development of colon cancers (8). Considering that IL-8 expression correlates with induction and progression of colorectal cancer, it would be important to understand the precise mechanism by which BFT induces IL-8 secretion (21). Taken together, our results suggest that in the HT29/C1 cell line, BFT induces IL-8 secretion by disruption of E-cadherin junctions and changes in cellular contacts which then lead to activation of the β-catenin pathway. BFT is known to induce E-cadherin cleavage and IL-8 secretion. However, it is not clear if E-cadherin cleavage is required for IL-8 secretion. Our data, raise the possibility that E-cadherin disruption and therefore cell-cell dissociation is sufficient to induce IL-8 secretion. We suggest that E-cadherin cleavage per se may not be required for BFT induced IL-8 secretion.

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## CONFLICT OF INTEREST

All authors have no financial conflict of interest.

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