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Brief Communication

Immunofluorescence Microscopic Evaluation of Tight Junctional Proteins during Enterotoxigenic *Bacteroides fragilis* (ETBF) Infection in Mice

Soonjae Hwang[1,*], Yeowool Kang[1,*], Minjeong Jo[1,*], Sung Hoon Kim[1,*], Won Gil Cho[2,] and Ki-Jong Rhee[1,†,**]**

[1]*Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University at Wonju, Wonju, Gangwon-do 26493, Korea*
[2]*Department of Anatomy, Wonju College of Medicine, Yonsei University at Wonju, Wonju, Gangwon-do 26493, Korea*

Inflammatory bowel disease (IBD) is increasing in prevalence in developed countries but the cause of this increase is unclear. In animal models of IBD and in human IBD patients, alterations in the tight junctional proteins have been observed, suggesting that the intestinal microflora may penetrate the underlying colonic tissue and promote inflammation. Enterotoxigenic *Bacteroides fragilis* (ETBF) causes inflammatory diarrhea in human and is implicated in inflammatory bowel diseases. However, it is unclear whether alterations in tight junctional proteins occur during ETBF infection in mice. In this brief communication, we report that ETBF infection induces up-regulation of claudin-2 and down-regulation of claudin-5 through *B. fragilis* toxin (BFT) activity in the large intestine of C57BL/6 mice. In contrast, BFT did not induce changes in tight junctional proteins in the HT29/C1 cell line, suggesting that analysis of biological activity of BFT *in vivo* is important for evaluating ETBF effects.

Key Words: Enterotoxigenic *Bacteroides fragilis*, Tight junction, Claudin, *Bacteroides fragilis* toxin

Intestinal epithelial cell permeability enhances communication between the intestinal microflora and the immune system (Hwang et al., 2018). Members of the tight junctional proteins, claudins and occludin, are the principal constituents of the epithelial tight junctions which regulate paracellular permeability in an intact epithelium (Chiba et al., 2008). In inflammatory bowel diseases (IBD) which include Crohn's disease (CD) and ulcerative colitis (UC), alterations in tight junctions have been shown. In CD patients, a robust up-regulation of claudin-2 was reported whereas down-regulation of claudin-4 was reported in UC patients (Prasad et al., 2005; Zeissig et al., 2007; Weber et al., 2008). Claudin-2 is unique among claudin family members as its normal expression is restricted to leaky epithelia *in vivo*. Claudin-4 plays a role in cation barrier formation and leads to increased transepithelial resistance and decreased Na^+ permeability (Van Itallie et al., 2001). Claudin-5 is predominantly expressed in endothelial tight junctions and is closely associated with vascular diseases (Burek et al., 2010). Deletion of claudin-7 led to the disruption of intestinal architecture with mucosal ulcerations, epithelial sloughing and inflammation (Tanaka et al., 2015). Claudin-15-deficient mice exhibited enlarged intestines in

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[*]Graduate student, [**]Professor.
[†]Corresponding author: Ki-Jong Rhee. Department of Biomedical Laboratory Science, College of Health Sciences, Yonsei University at Wonju, Wonju, Gangwon-do 26493, Korea.
Tel: +82-33-760-2445, Fax: +82-33-760-2195, e-mail: kjrhee@yonsei.ac.kr
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which the upper small intestine was larger than the normal intestine, indicating a pivotal role of claudin-15 in modulating morphogenesis of the small intestine of mice (Tamura et al., 2008). Gut integrity is mainly affected by gut microbes which may increase or decrease gut leakiness. In *Salmonella thyphimurium*-infected mice, claudin-2 was up-regulated in the colon (Zhang et al., 2013). In contrast, it has also been shown that *S. thermophilus* and *L. acidophilus* could protect against EIEC infection-induced alterations in integrity of tight junction in colonic epithelial cells (Resta-Lenert and Barrett, 2003).

Bacteroides fragilis are normal colonic commensals in the majority of adults (Kim et al., 2016; Gwon et al., 2015). A molecular subset of *B. fragilis*, termed enterotoxigenic *B. fragilis* (ETBF), secretes a 20-kDa zinc-dependent metalloprotease toxin called *B. fragilis* toxin (BFT) that reversibly stimulates chloride secretion and alters adherent junctional function in polarized intestinal epithelial cells (Jang et al., 2015). The main activity of BFT is the induction of E-cadherin cleavage in colonic epithelial cell lines. In addition, it was reported that ETBF-induced colonic inflammation is mediated by BFT in mice and pathology of IBD rodent model is aggravated by ETBF colonization (Rabizadeh et al., 2007; Rhee et al., 2009). ETBF-induced infection is characterized by Th17 immune response in large intestine of mice (Wu et al., 2009). It was reported that Th17 immune response-related cytokines are strongly associated with IBD pathogenesis and modulate expression of claudins in gut (Capaldo and Nusrat, 2009; Liu et al., 2009; Eastaff-Leung et al., 2010; Neurath, 2014). However, ETBF-induced alterations in tight junctional proteins has not been comprehensively investigated *in vivo*. In this study, we investigated whether ETBF colonization in mice modified tight junction in colon of mice.

Six-week old, female C57BL/6 mice (total of 7~10 per group) were orally inoculated with 1×10^9 CFU of recombinant ETBF (rETBF) (strain NCTC 9343 transformed with pFD340 carrying *bft-2* gene), recombinant-nontoxigenic *B. fragilis* (rNTBF) (strain NCTC 9343 transformed with pFD340 carrying mutated, inactive *bft-2* H352Y gene) as described previously (Wu et al., 2007a; Rhee et al., 2009). There were no significant differences in *B. fragilis* coloni-

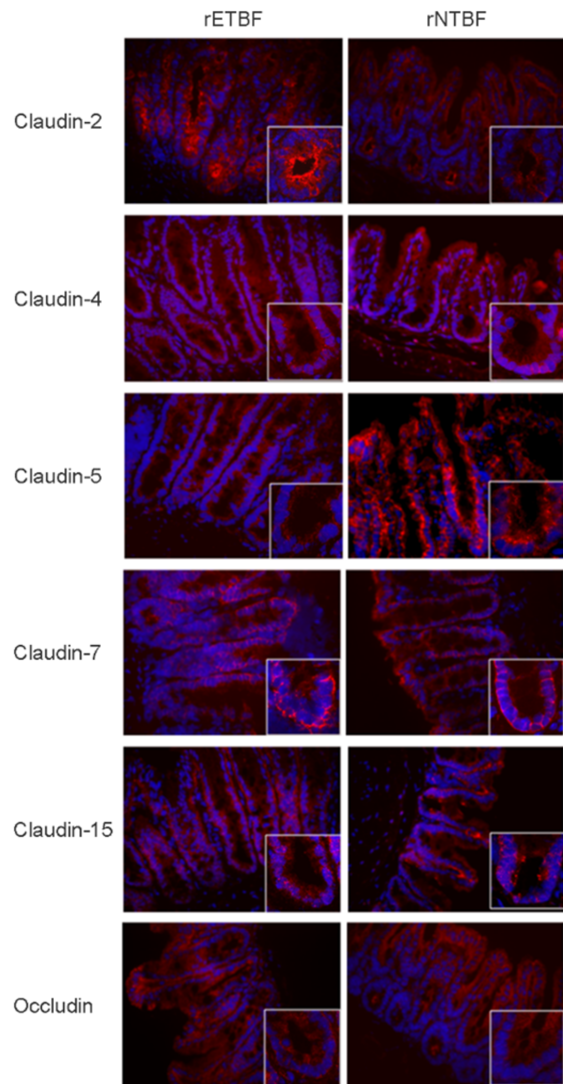

Fig. 1. Representative immunoflourescence-stained images of ceca from mice infected with rETBF and rNTBF. Prior to infection, 6-week-old, female C57BL/6 mice were given drinking water containing clindamycin and gentamicin for 5 days to promote *B. fragilis* colonization and for consistency of infection. C57BL/6 mice (total of 7~10 per group) were orally inoculated with 1×10^9 CFU of recombinant ETBF (rETBF) (strain NCTC 9343 transformed with pFD340 carrying *bft-2* gene), recombinant-nontoxigenic *B. fragilis* (rNTBF) (strain NCTC 9343 transformed with pFD340 carrying mutated, inactive *bft-2* H352Y gene). At 7 days post-infection, ceca were embedded in OCT compound and cryosectioned (7 μm), followed by fixation of slides in 10% acetone for 20 minutes. Nonspecific binding of antibodies was inhibited by blocking with PBS containing 5% inactivated goat serum. Each section was incubated with primary antibodies to claudin-2 (Abcam, ab53032), claudin-4 (3E2C1, Invitrogen, 329494), claudin-5 (Invitrogen, 34-1600), claudin-7 (Invitrogen, 34-9100), claudin-15 (Invitrogen, 38-9200) and occludin (Invitrogen, 71-1500). Appropriate Cy3-labelled secondary antibodies were used. Counterstaining of cell nuclei was performed with DAPI. Images were obtained using a Nikon immunofluorescent microscope and Adobe Photoshop software was used for processing of images. Magnification X400, digital magnification of select fields (insets).

zation between rETBF-infected mice and rNTBF-infected mice (data not shown). Experimental design was approved by the Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC) of Yonsei Wonju University (IBC approval number; 201612-P-014-01, IACUC approval number; YWCI-201612-014-01). Furthermore, there were no clinical nor histologic differences between rNTBF-inoculated and PBS-inoculated group. Therefore, we showed data for only the rETBF-infected mice and rNTBF-infected mice. At 7 days post-infection ceca were stained with primary antibodies to claudin-2, claudin-4, claudin-5, claudin-7, claudin-15 and occludin (see Fig. 1 legend for details). We found that rETBF colonized mice showed high levels of claudin-2 intensity in the base of crypt of cecum compared with rNTBF colonized mice (Fig. 1). In contrast, claudin-5 showed down-regulated levels in cecum of rETBF colonized mice compared to cecum of rNTBF colonized mice. Significant alterations of other claudins (claudin-4, claudin-7, claudin-11) and occludin were not observed in cecum of both groups.

It was reported that activated Wnt/β-catenin pathway increases claudin-2 expression in mouse mammary epithelial

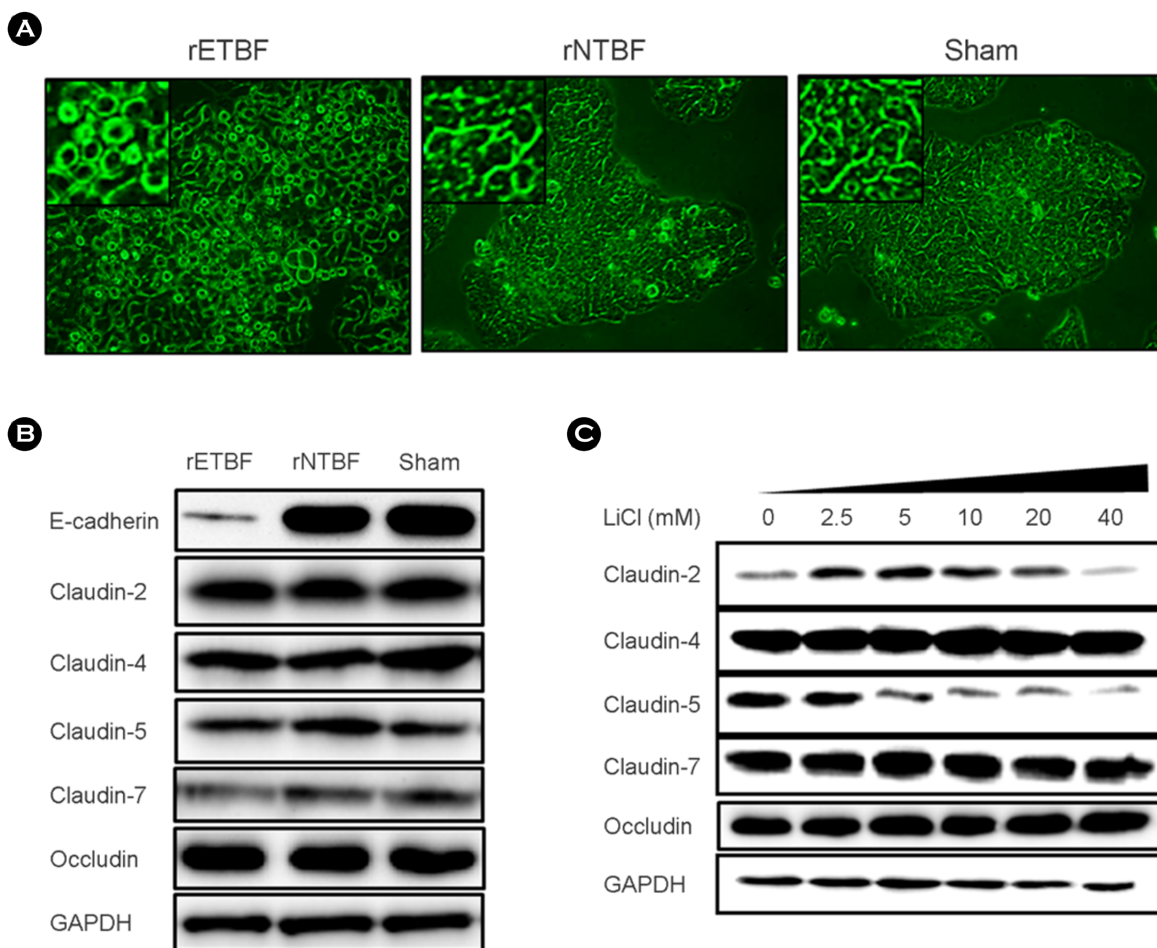

Fig. 2. Western blot analysis of HT29/C1 cells treated with BFT or LiCl. (A) Green-filtered microscopic images. Magnification X400 (Left upper panels, digital magnification of select fields). (B) Western blot of HT29/C1 cells treated with active BFT, inactive BFT and serum-free media (sham) for 24 hours. To exclude effects of bacterial cells, the bacterial supernatant was filtered using a syringe filter (0.45 μm). The HT29/C1 cells were subconfluently seeded on 6-well plates (1×10^5 cell per well) in 10% FBS-DMEM and grown until 80~90% confluency. The FBS-containing DMEM was exchanged with serum-free DMEM in order to prevent neutralization of BFT by serum proteins. The HT29/C1 cells were then treated with rETBF and rNTBF supernatants containing active and inactive BFT, respectively, for 24 hr. Thereafter, cell lysates were prepared using RIPA buffer. Western blot was performed using appropriate primary and secondary antibodies. (C) Western blot of HT29/C1 cells treated with LiCl in a dose-dependent manner for 24 hr.

C57 cells (Mankertz et al., 2004) and decreases claudin-5 expression in endothelial cells (Taddei et al., 2008). In addition, BFT induces activation of Wnt/β-catenin pathway in a colonic epithelial cell line of HT29/C1 (Wu et al., 2003). Therefore, we hypothesized that BFT directly up-regulates claudin-2 and down-regulates claudin-5 in colonic epithelial cells. The human colonic adenoma cell line HT29/C1 is the most often used cell line to examine the effects of BFT. BFT-treated HT29/C1 cells showed morphologic traits such as cell rounding and detachment, all indicative of active BFT effects on HT29/C1 cells (Chambers et al., 1997; Wu et al., 2003; Wu et al., 2007b; Hwang et al., 2013). HT29/C1 cells were treated with rETBF or rNTBF bacterial supernatants for 24 hr and Western blot analysis was conducted for various tight junctional proteins. We confirmed that the rETBF supernatant containing active BFT-induced the morphologic alterations and E-cadherin cleavage in HT29/C1 cells (Fig. 2A). Contrary to our expectations, protein levels of all claudins (claudin-2, -4, -5, -7) and occludin remained unchanged in both rETBF and rNTBF treated groups (Fig. 2B). We found no changes in proteins levels of claudins and occludin at different time points (6 h, 12 h, 48 h) (data not shown). To determine whether changes in claudin can be observed under other conditions, HT29/C1 cells were treated with lithium chloride (LiCl) to activate Wnt/β-catenin pathway. We confirmed that LiCl-treated HT29/C1 cells showed increased claudin-2 protein and decreased claudin-5 protein levels (Fig. 2C). There was also no alteration in levels of claudin-4, claudin-7 and occludin in the LiCl-treated cells (Fig. 2C). These results suggest that BFT does not induce changes in tight junction protein, albeit in a cell line. In summary, rETBF colonization induced alterations of claudin-2 and claudin-5 in large intestine of C57BL/6 mice compared with rNTBF colonized group, which suggests that changes in claudin-2 and clauin-5 were attributed to BFT activity. However, BFT did not induce alterations in claudins (claudin-2, -4, -5, -7) and occludin *in vitro*. In conclusion, we report that active BFT secreted by ETBF modulates claudin-2 and claudin-5 levels in the large intestine of mice. This finding was not recapitulated in the HT29/C1 cells line, emphasizing the importance of evaluating biologically activity of BFT *in vivo*.

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CONFLICT OF INTEREST

No conflict of interests exists for any of the authors.

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