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Bacteroides fragilis Toxin Induces Cleavage and Proteasome Degradation of E-Cadherin in Human Breast Cancer Cell Lines BT-474 and MCF7

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Abstract

Enterotoxigenic *Bacteroides fragilis* (ETBF) has been reported to promote colitis and colon cancer through the secretion of *B. fragilis* toxin (BFT), a zinc-dependent metalloprotease. In colonic epithelial cells, BFT induces the cleavage of E-cadherin into the 80 kDa ectodomain and the 33 kDa membrane-bound intracellular domain. The resulting membrane-tethered fragment is then cleaved by γ-secretase forming the 28 kDa E-cadherin intracellular fragment. The 28 kDa cytoplasmic fragment is then degraded by an unknown mechanism. In this study, we found that the 28 kDa E-cadherin intracellular fragment was degraded by the proteasome complex. In addition, we found that this sequential E-cadherin cleavage mechanism is found not only in colonic epithelial cells but also in the human breast cancer cell line, BT-474. Lastly, we reported that staurosporine also induces E-cadherin cleavage in the human breast cancer cell line, MCF7, through γ-secretase. However, further degradation of the 28 kDa E-cadherin intracellular domain is not dependent on the proteasome complex. These results suggest that the BFT-induced E-cadherin cleavage mechanism is conserved in both colonic and breast cancer cells. This observation indicates that ETBF may also play a role in the carcinogenesis of tissues other than the colon.

Keywords

Enterotoxigenic *Bacteroides fragilis*
E-cadherin
Proteasome
Staurosporine

1. Introduction

Bacteroides fragilis (*B. fragilis*) is a Gram-negative, facultatively anaerobic bacterium that is present in the human gut flora [1]. A subtype of *B. fragilis*, enterotoxigenic *B. fragilis* (ETBF), is a known enteric pathogen that causes diarrhea and colitis in livestock

and humans [2]. The primary pathogenesis of ETBF is caused by a 20 kDa metalloprotease called *Bacteroides fragilis* toxin (BFT), which cleaves the ectodomain of the junctional protein E-cadherin in colonic epithelial cells [3]. E-cadherin cleavage by BFT induces an inflammatory response in intestinal epithelial cells through the nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and β-catenin signaling systems [4]. BFT also acts as a potential carcinogen in the pathogenesis of colorectal cancer, inducing epigenetic changes and deoxyribonucleic acid (DNA) damage in intestinal epithelial cells [5,6].

Recently, the downstream effects of E-cadherin cleavage have been investigated in relation to cancer development [7]. E-cadherin is a 120 kDa glycoprotein located on the lateral surface of all epithelial cells and mediates the adhesion of neighboring cells by homophilic interactions [8]. The intracellular domain of E-cadherin forms an E-cadherin-catenin complex that binds to the actin cytoskeleton to maintain cell shape, polarity, and physiological functions such as migration and differentiation [9]. Cleavage of E-cadherin induces cellular pathophysiological states and is considered a tumor suppressor protein in that its loss can induce epithelial-mesenchymal transition (EMT) in cancer metastasis [10]. Therefore, understanding the mechanisms of E-cadherin cleavage is important to elucidate the cellular physiology mediated by E-cadherin. Cleavage of E-cadherin was first detected in breast cancer cells [11]. Staurosporine, a non-specific protein kinase inhibitor, is known to induce γ-secretase-dependent cleavage of the intracellular domain of E-cadherin in human breast cancer cell lines T47D and MCF7 cells [12]. Staurosporine promotes EMT through upregulation of matrix metalloproteinase (MMP)-2 in the PMC42-LA human breast cancer cell line [13].

The mechanism of BFT-induced E-cadherin cleavage was mainly studied using the human colon cancer cell line, HT29/C1 cells [14]. Active BFT cleaves 120 kDa full-length E-cadherin to produce an 80 kDa E-cadherin ectodomain and a 33 kDa E-cadherin intracellular domain. It is not known whether BFT binds directly to E-cadherin or activates a protease that cleaves E-cadherin. BFT-induced E-cadherin cleavage was also induced by small interfering ribonucleic acid (siRNA) of proteases such as MMP-7, and a disintegrin and metalloproteinase 10. After cleavage of the E-cadherin ectodomain, cell membrane γ-secretase cleaves the intracellular domain to produce a 5 kDa juxtamembrane domain and a 28 kDa intracellular fragment. It is known that intracellular proteases such as calpain, caspase-3, and proteasome are involved in the degradation of E-cadherin cytoplasmic domain [7,15,16]. However, the mechanism of degradation of the 28 kDa intracellular fragment induced by BFT is still unknown (**Figure 1**).

Proteasome is a protein complex involved in physiological activities including transcription, cell cycle, apoptosis, cell metabolism, and oxidative stress [17,18]. The main function of the proteasome is to degrade damaged or misfolded proteins that are not required for cellular homeostasis [17]. In particular, transmembrane proteins such as junctional proteins and integral receptors are regulated by the ubiquitin-proteasome system [19]. Proteasomes have been reported to be involved in EMT of tumor metastasis by regulating E-cadherin expression [20]. Cytoplasmic E-cadherin has a mouse double minute 2 (MDM2) (E3 ubiquitin ligase) binding site, suggesting that it may be a substrate for proteasome [21]. In this study, we used bortezomib and carfilzomib, inhibitors of the dipeptide boronate family, to inhibit the proteasome. Bortezomib is a reversible inhibitor that binds to the chymotrypsin-like and caspase-like sites of the β5 subunit of the proteasome and various serine proteases, while carfilzomib is an irreversible inhibitor that binds to the chymotrypsin-like site of the β5 subunit [22,23].

In this study, we confirmed that the 28 kDa E-cadherin fragment generated by BFT is degraded by the proteasome. We aimed to determine whether BFT-induced E-cadherin fragmentation that occurs in colon cancer cells also occurs in breast cancer cells using the human breast cancer cell line BT-474 cells. We also

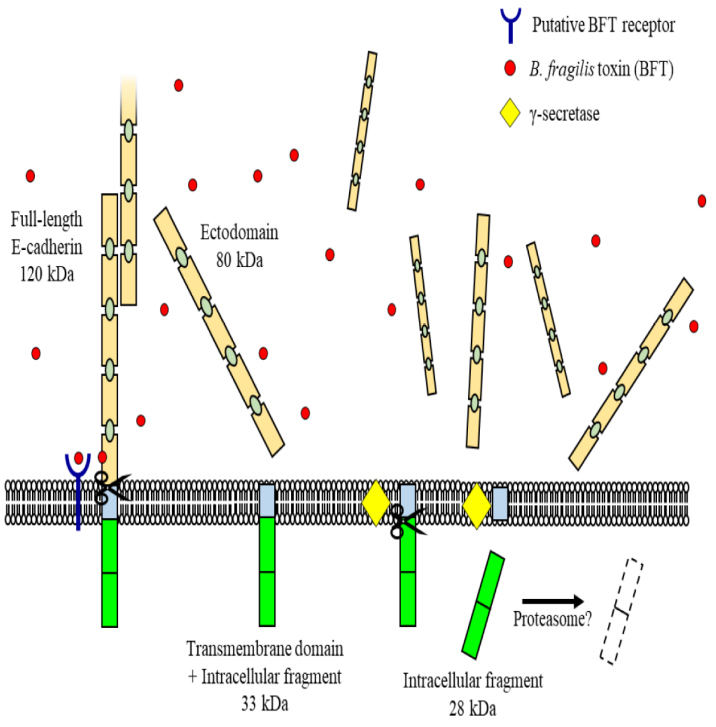

Figure 1. Schematic diagram of E-cadherin cleavage by BFT. In HT29/C1 colonic epithelial cells, BFT induces ectodomain cleavage of the full-length E-cadherin (120 kDa) by an unknown mechanism. The resulting E-cadherin ectodomain (80 kDa) is shed into the cell culture media. The membrane-tethered E-cadherin fragment (33 kDa) is then cleaved by γ-secretase resulting in the release of the E-cadherin intracellular fragment (28 kDa) into the cytoplasm. We hypothesize that the degradation of the 28 kDa fragment is due to the proteasome complex. Whether BFT binds to a putative BFT receptor or directly cleaves E-cadherin is also unknown. Abbreviation: BFT, *Bacteroides fragilis* toxin.

wanted to compare E-cadherin cleavage mediated by staurosporine and BFT.

2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines (BT-474, MCF7) and colon cancer cell lines (HT29/C1) used in this study were cultured in Dulbecco's modified Eagle's medium (Corning Inc, Corning, NY, USA) supplemented with 5% fetal bovine serum (Gibco, Carlsbad, CA, USA), 10 mM HEPES (Gibco, Carlsbad, CA, USA), penicillin (100 U/mL) / streptomycin (100 μg/mL) (Gibco, Carlsbad, CA, USA), and cultured in a 37°C, 5% CO_2 incubator.

2.2. Strain and bacterial culture

Nontoxigenic wild-type *B. fragilis* (WT-NTBF) overexpressing active BFT (rETBF; bft-2) and WT-NTBF overexpressing inactive mutant BFT (rNTBF; bft-2 H352Y) were kindly provided by Cynthia Sears and Augusto Franco (Johns Hopkins University, Baltimore, MA, USA). All *Bacteroides* strains used in this study are naturally resistant to gentamicin and were transformed with pFD340, which confers clindamycin resistance. The two recombinant *Bacteroides* strains (rETBF, rNTBF) were inoculated on brain heart infusion agar supplemented with hemin, vitamin K1, cysteine, and two antibiotics (clindamycin and gentamicin), and then incubated in an anaerobic chamber at 37°C for 48 hours. Single colonies were then picked and inoculated into brain heart infusion broth (BHIB) and incubated in an anaerobic chamber at 37°C for 48 hours. The bacterial supernatant was centrifuged and filtered using a 0.45 μm syringe filter (Sartorius, Goettingen, Germany) and stored at -80°C.

2.3. Inhibitor treatment

Cells used in this study were pretreated with 1.5 μM γ-secretase inhibitor (L-685,458; Calbiochem, San Diego, CA, USA) or 2 μM proteasome inhibitor (bortezomib or carfilzomib; Focus Biomolecules, Plymouth Meeting, PA, USA) for 30 minutes. 250 μM non-specific protein kinase inhibitor staurosporine (Sigma Aldrich, St. Louis, MO, USA) was used for treatment. All inhibitors were added

to serum-free media and treated for the indicated period, along with a 1:20 dilution of BHIB, rNTBF (rNT) or rETBF (rET) supernatants.

2.4. Western blot analysis

Western blot was performed to investigate E-cadherin cleavage induced by BFT and staurosporine. Cells were washed with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA; Sigma Aldrich) buffer supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktail (Sigma Aldrich) for 10 minutes at 4℃. Cell lysates and supernatants were centrifuged (12,000 RCF) at 4°C for 10 minutes, and the supernatant was used. Proteins were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membrane (Pall, Washington, NY, USA). The membrane was blocked with double blocker (T&I, Chuncheon, Korea) for 10 minutes at room temperature. As primary antibodies, E-cadherin monoclonal antibody (C36, 1:2000; BD Biosciences, San Jose, CA, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 6C5, 1:2,000, Sigma Aldrich) were used overnight at 4℃. After washing five times with Tris-buffered saline with tween 20 (TBST), the sections were reacted with the secondary antibody horseradish peroxidase (HRP) labeled anti-mouse immunoglobulin G (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour at room temperature. After washing with TBST, protein bands were identified using an enhanced chemiluminescence (ECL) kit (Bio-Rad, Hercules, CA, USA).

3. Result

3.1. Degradation of a 28 kDa E-cadherin fragment by the proteasome

To investigate whether the proteasome degrades the 28 kDa E-cadherin intracellular fragment, HT29/C1 cells, a human intestinal epithelial cell line, were pretreated with the proteasome inhibitors bortezomib and carfilzomib for 30 minutes. The rNTBF supernatant (rNT), which secretes an inactive BFT, and the rETBF supernatant (rET), which secretes an active BFT, were treated with the proteasome inhibitors together. The 120 kDa full-length E-cadherin was not cleaved by BHIB or rNT, and cleavage of the 120 kDa full-length E-cadherin was observed starting at 10 minutes of rET. When treated with BFT alone, rET produced 33 kDa and 28 kDa E-cadherin fragments, but the amount of E-cadherin decreased over time. When treated with bortezomib, the 33 kDa E-cadherin intracellular domain was cleaved by γ-secretase and decreased, but the proteasome was inhibited, resulting in the accumulation of the 28 kDa E-cadherin intracellular fragment (**Figure 2A**). Carfilzomib also inhibited the degradation of the 28 kDa E-cadherin cytoplasmic fragment in a time-dependent manner, similar to bortezomib (**Figure 2B**). These results suggest that the 28 kDa E-cadherin fragment generated by BFT was degraded by the proteasome.

3.2. E-cadherin fragmentation in the breast cancer cell line BT-474

To investigate whether BFT induces E-cadherin fragmentation in breast cancer cells as well as colon cancer cells, a breast cancer cell line, BT-474 cells, were treated with BFT and γ-secretase inhibitors (L-685,458) and proteasome inhibitors (bortezomib, carfilzomib). BT-474 cells were pretreated with γ-secretase inhibitors and proteasome inhibitors for 30 minutes, and then BFT was treated with the inhibitors. In BT-474 cells, BFT induced the cleavage of E-cadherin, significantly reducing the 120 kDa full-length E-cadherin within 30 minutes, and confirmed the accumulation of the 33 kDa E-cadherin intracellular domain by the γ-secretase inhibitor (**Figure 3A**). Furthermore, treatment with the proteasome inhibitors bortezomib and carfilzomib decreased the 33 kDa intracellular domain but increased the 28 kDa E-cadherin intracellular fragment (**Figure 3B, 3C**).

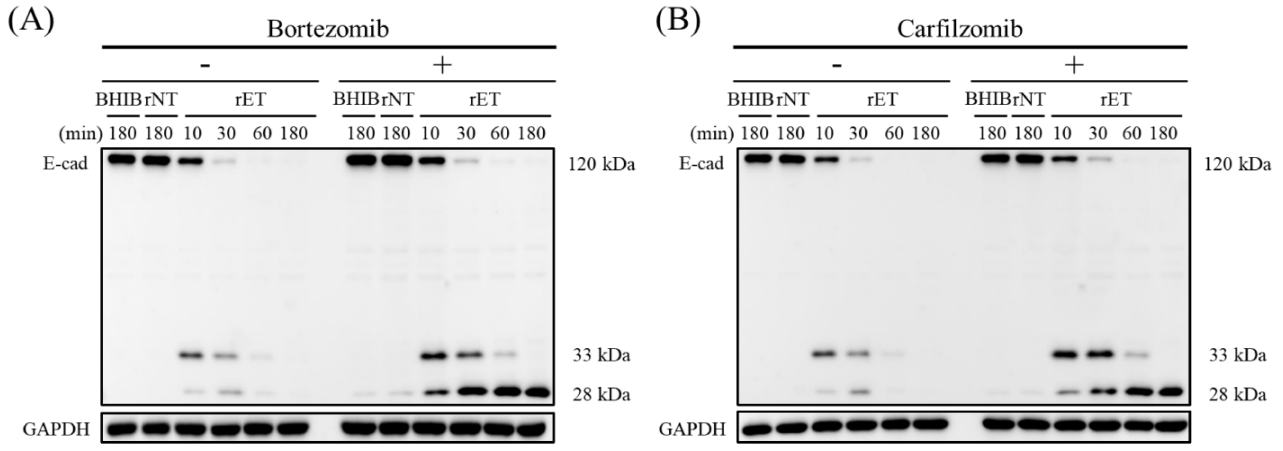

Figure 2. *Bacteroides fragilis* toxin-induced 28 kDa E-cadherin intracellular fragment is degraded by the proteasome complex in HT29/C1 cells. Colonic epithelial HT29/C1 cells were pretreated with proteasome inhibitors, (A) bortezomib (2 μM) or (B) carfilzomib (2 μM) for 30 minutes and incubated with BHIB, rNT or rET for the indicated duration (10, 30, 60, and 180 minutes). Cell lysates were examined by Western blot analysis using C-terminal specific E-cadherin antibodies. GAPDH was used as internal control. Abbreviations: BHIB, brain heart infusion broth; rNT, rNTBF supernatant; rET, rETBF supernatant; E-cad, E-cadherin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

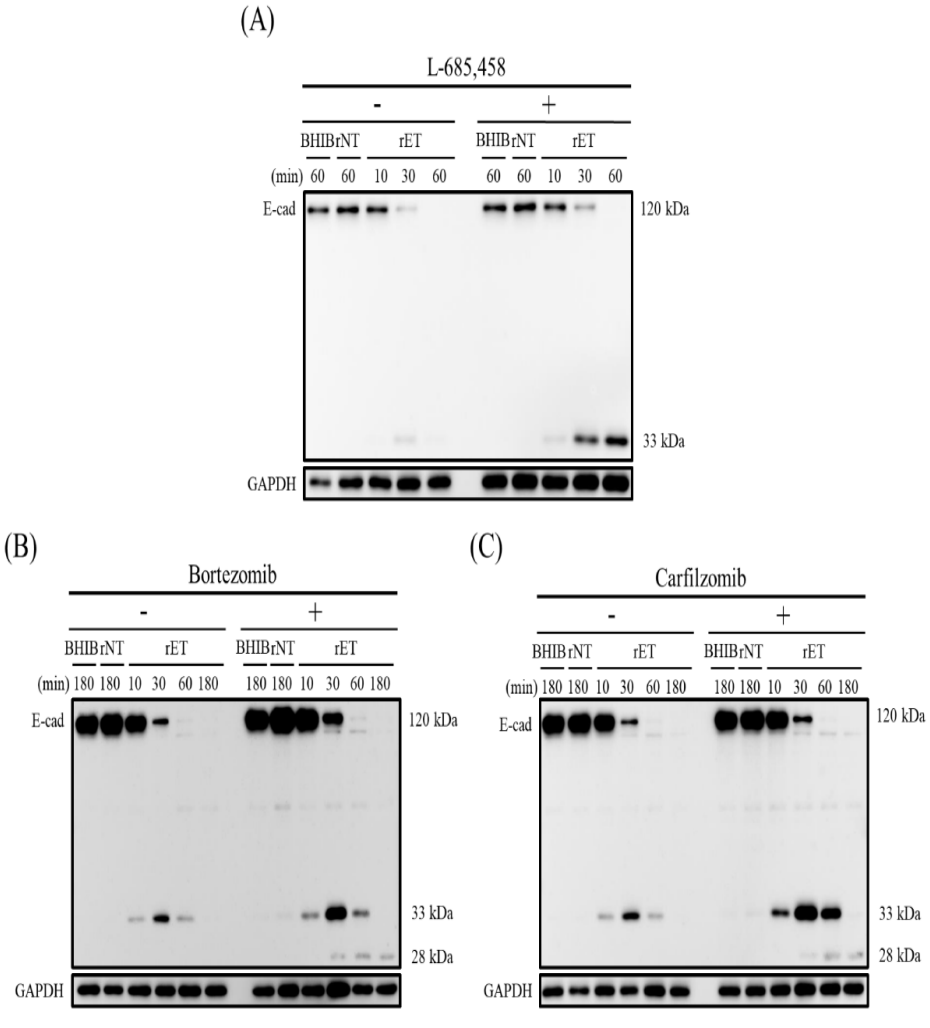

Figure 3. *Bacteroides fragilis* toxin induces sequential cleavage of E-cadherin and degradation of the 28-kDa E-cadherin intracellular fragment in BT-474 cells. Human breast epithelial BT-474 cells were pretreated with (A) γ-secretase inhibitor (L-685,458; 1.5 μM) or proteasome inhibitor, (B) bortezomib (2 μM), or (C) carfilzomib (2 μM) for 30 minutes and incubated with BHIB, rNT or rET for indicated duration (10, 30, 60, and 180 minutes). Cell lysates were examined by Western blot analysis using C-terminal specific E-cadherin antibodies. GAPDH was used as internal control. Abbreviations: See **Figure 2**.

3.3. Comparison of E-cadherin fragmentation induced by staurosporine and BFT

To compare BFT-induced E-cadherin fragmentation with staurosporine-induced E-cadherin fragmentation, human breast cancer cell line MCF7 cells were treated with staurosporine, BFT, a γ-secretase inhibitor, and a proteasome inhibitor. The γ-secretase inhibitor and proteasome inhibitor were pretreated with MCF7 cells for 30 minutes, followed by treatment with staurosporine or BFT in combination with the inhibitor. The morphology of MCF7 cells is adherent cells with cell polarity, and treatment with BFT did not cause them to lose their polarity, and they did not appear to lose their connections with neighboring cells. On the other hand, when treated with staurosporine, MCF7 cells lost their polarity and became rounded (**Figure 4A**). When staurosporine and BFT were treated for 3 and 6 hours, BFT induced a significant decrease in E-cadherin, but staurosporine did not induce a significant decrease, and treatment with γ-secretase and proteasome inhibitors did not result in an increase in 33 kDa or 28 kDa E-cadherin in the E-cadherin cleavage by staurosporine. In contrast, BFT increased 33 kDa and 28 kDa E-cadherin when treated with γ-secretase and proteasome inhibitors (**Figure 4B, 4C**). When staurosporine and BFT were treated for 9 hours, staurosporine induced E-cadherin cleavage but cleaved less E-cadherin than BFT. In addition, accumulation of the 33 kDa intracellular domain by the γ-secretase inhibitor, but not the 28 kDa intracellular fragment by the proteasome inhibitor, occurred with staurosporine (**Figure 4D**).

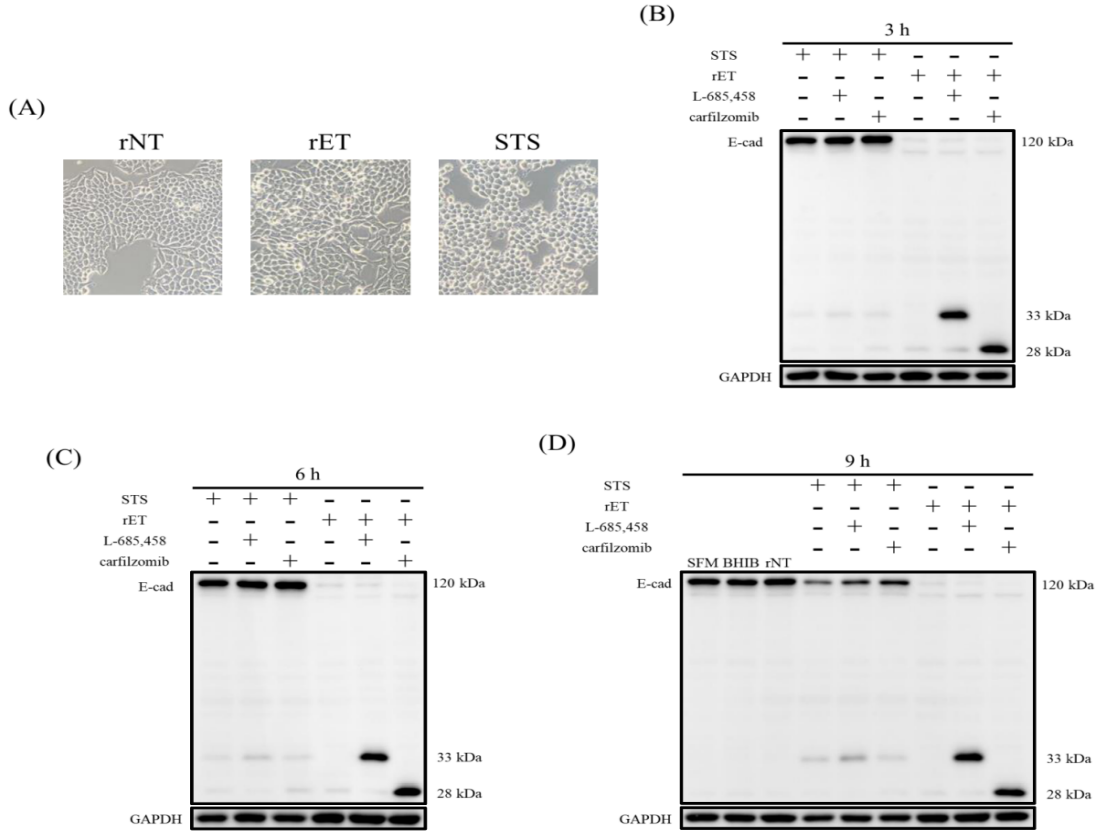

Figure 4. Cell morphology changes and E-cadherin cleavage kinetics are different in staurosporine and *Bacteroides fragilis* toxin-treated MCF7 cells. (A) Human breast MCF7 cells were treated with rET, 250 μM STS, SFM, BHIB, or rNT for 9 hours and cellular images were taken by using an inverted microscope (×40). MCF7 cells were pretreated with 1.5 μM γ-secretase inhibitor (L-685,458) or 2 μM proteasome inhibitor (carfilzomib) for 30 minutes, and incubated with 250 μM STS or rET for the indicated duration, 3 hours (B), 6 hours (C), or 9 hours (D). Cell lysates were examined by Western blot analysis using C-terminal specific E-cadherin antibodies. GAPDH was used as internal control. Abbreviations: See **Figure 2**; STS, staurosporine; SFM, serum-free media.

4. Discussion

ETBF has been implicated in inflammatory bowel disease or colitis-associated cancer [24]. ETBF-secreted BFT activates the β-catenin/NF-κB and MAPK signaling pathways via E-cadherin cleavage, leading to increased levels of interleukin-8, a cytokine that is elevated in acute and chronic inflammation [4,25]. Since ETBF is an enterobacterium typically residing in the large intestine, studies to elucidate its mechanisms have primarily been conducted in intestinal epithelial cells or animal models of inflammatory colon cancer [26,27]. However, a few gut bacteria have been found in organs such as the liver, breast, or spleen, where they can induce pathophysiologic responses [28,29]. A recent study reported that intraductal injection of ETBF into the mammary gland of mice resulted in the formation of ETBF colonies with mammary epithelial cell proliferation, local inflammation, and tissue fibrosis [30]. In addition, knockdown of E-cadherin by short hairpin RNA (shRNA) in the human breast cancer cell line MCF7 resulted in an 8-fold increase in cell motility and a 3-fold increase in invasiveness [31]. Therefore, in this study, we determined whether E-cadherin was cleaved by BFT in BT-474 cells. Similar to HT29/C1 cells, cleavage of E-cadherin by BFT in BT-474 cells was induced and sequentially degraded by γ-secretase and proteasome. These results suggest that ETBF infection may have pathological effects not only in the intestine but also in other organs.

The ubiquitin-proteasome system mainly degrades proteins with short solubility or lifespan and misfolded proteins, while the lysosome mainly degrades long-lived proteins, protein aggregates, and damaged organelles [32,33]. As previous studies have suggested that E-cadherin can be degraded by both proteasome and lysosome [34,35], we used MG132, the most common proteasome inhibitor, as a preliminary study to inhibit the proteasome. MG132, similar to bortezomib and carfilzomib, is known to inhibit the proteasome by binding to the chymotrypsin-like site of the proteasome β5 subunit [36]. In our previous study, MG132 inhibited both proteasome and γ-secretase at concentrations above 4 μM in a human breast cancer cell line (MCF-7) (data not shown). In this context, MG132 is not suitable for mechanism studies involving γ-secretase and proteasome. In this study, we found that specific inhibition of the proteasome with the proteasome inhibitors bortezomib and carfilzomib in HT29/C1 cells inhibited the degradation of a 28 kDa intracellular fragment. However, this study only identified E-cadherin degradation involving the proteasome, and it is necessary to study whether the lysosome is involved in the mechanism of E-cadherin cleavage in the future.

Previous studies have shown that staurosporine induced cleavage of E-cadherin in human breast cancer cell lines (H184A1, MCF7, T47D) and canine renal epithelial cell line (Madin-Darby canine kidney, MDCK) [12,34], and BFT cleaved E-cadherin in HT29/C1 cells in 30 minutes and induced morphological changes [37]. In this study, we compared staurosporine-induced E-cadherin cleavage with BFT-induced E-cadherin cleavage. Staurosporine altered the morphology of MCF7 cells after 3 hours of treatment, but 120 kDa E-cadherin was still present at 9 hours of treatment. In contrast, BFT maintained polarity with no change in cell morphology after 9 hours of treatment. Whereas E-cadherin was mostly reduced after 3 hours of BFT treatment, E-cadherin was still present at 9 hours of staurosporine treatment. In staurosporine-induced E-cadherin cleavage, γ-secretase cleaved the E-cadherin intracellular domain, but the proteasome was not involved in the degradation. These conflicting results suggest that staurosporine-induced E-cadherin cleavage and BFT-induced E-cadherin cleavage have different mechanisms. Future studies should investigate whether staurosporine cleaves junctional proteins other than E-cadherin and the mechanisms of degradation of the resulting intracellular fragments.

Loss of E-cadherin is essential for EMT and is an important characteristic of metastatic cells. BFT disrupts the adherens junctions of epithelial

cells, allowing adherent cells to detach and migrate elsewhere freely [38]. In this context, the study of E-cadherin cleavage mechanisms will contribute to the understanding of bacteria-induced EMT as well as cancer metastasis in general.

5. Conclusion

Enterotoxigenic *Bacteroides fragilis* (ETBF), which causes inflammatory bowel disease and colon cancer, secretes *B. fragilis* toxin (BFT), a zinc-dependent metalloprotease. BFT induces cleavage of epithelial cell E-cadherin into an 80 kDa ectodomain and a 33 kDa intracellular domain. The resulting E-cadherin intracellular domain is sequentially cleaved by γ-secretase, and the 28 kDa E-cadherin intracellular fragment is degraded by a mechanism that remains to be elucidated. In this study, we confirmed that the 28 kDa E-cadherin intracellular fragment resulting from BFT-induced E-cadherin cleavage is degraded by the proteasome. We also confirmed that the same mechanism of BFT-induced E-cadherin cleavage occurs in the human breast cancer cell line BT-474, but not in colon cancer cells. Lastly, staurosporine induced cleavage of E-cadherin in the human breast cancer cell line MCF7, by γ-secretase rather than proteasome. These results suggest that BFT-induced cleavage of E-cadherin can occur in the breast in addition to the colon, where ETBF resides, and that ETBF may be involved in cancers other than colon cancer.

Disclosure statement

The authors declare no conflict of interest.

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