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HOST RESPONSE AND INFLAMMATION

Intestinal Epithelial Cells Exposed to *Bacteroides fragilis* Enterotoxin Regulate NF-κB Activation and Inflammatory Responses through β-Catenin Expression

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ABSTRACT The *Bacteroides fragilis* enterotoxin (BFT), a virulence factor of enterotoxigenic *B. fragilis* (ETBF), interacts with intestinal epithelial cells and can provoke signals that induce mucosal inflammation. Although β-catenin signaling is reported to be associated with inflammatory responses and BFT is known to cleave E-cadherin linked with β-catenin, little is known about the β-catenin-mediated regulation of inflammation in ETBF infection. This study was conducted to investigate the role of β-catenin as a cellular signaling intermediate in the induction of proinflammatory responses to stimulation of intestinal epithelial cells with BFT. Expression of β-catenin in intestinal epithelial cells was reduced relatively early after stimulation with BFT and then recovered to normal levels relatively late after stimulation. In contrast, phosphorylation of β-catenin in BFT-exposed cells occurred at high levels early in stimulation and decreased as time passed. Concurrently, late after stimulation the nuclear levels of β-catenin were relatively higher than those early after stimulation. Suppression of β-catenin resulted in increased NF-κB activity and interleukin-8 (IL-8) expression in BFT-stimulated cells. However, suppression or enhancement of β-catenin expression neither altered the phosphorylated IκB kinase α/β complex nor activated activator protein 1 signals. Furthermore, inhibition of glycogen synthase kinase 3β was associated with increased β-catenin expression and attenuated NF-κB activity and IL-8 expression in BFT-exposed cells. These findings suggest the negative regulation of NF-κB-mediated inflammatory responses by β-catenin in intestinal epithelial cells stimulated with BFT, resulting in attenuation of acute inflammation in ETBF infection.

KEYWORDS *Bacteroides fragilis*, enterotoxin, intestinal epithelial cells, NK-κB, β-catenin

Enterotoxigenic *Bacteroides fragilis* (ETBF) is associated with intestinal diseases, such as colitis, inflammatory bowel disease, and colorectal cancer (1–3). The important cause of these diseases is known to be the enterotoxin produced by ETBF strains (2, 4). Exposure of intestinal epithelial cells to *B. fragilis* enterotoxin (BFT) rapidly activates nuclear transcriptional factors, such as nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1), leading to the release of proinflammatory mediators, such as interleukin-8 (IL-8) (5–8). We previously found that the activated signals of NF-κB and AP-1 in BFT-exposed intestinal epithelial cells gradually decline after exposure (5–8). Therefore, it is possible that some factors may modulate the activities of transcriptional factors in BFT-exposed cells and contribute to the regulation of enteric inflammation.

Although ETBF strains are considered enteric pathogens, clinical studies have revealed that in many cases of infection, bacteria alone are present without symptoms of enteritis (4, 8, 9). Therefore, it is believed that some negative regulatory signals for

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enteric inflammation might be induced after intestinal epithelial cells are exposed to BFT derived from ETBF. In the present study, we propose that altered expression of β-catenin is one of these regulatory signals.

β-Catenin is a member of the Wnt/β-catenin pathway, which regulates various cellular processes, such as cellular proliferation, differentiation, and development, as well as intercellular adhesion (10–12). In the absence of extracellular Wnt ligands, the canonical Wnt/β-catenin pathway is inactive (Wnt-off state) and β-catenin is maintained at low levels in the cytoplasm due to its degradation through the ubiquitin-proteasome pathway. The β-catenin destruction complex is formed by the scaffold protein axin, adenomatous polyposis coli protein (APC), glycogen synthase kinase 3β (GSK-3β), and casein kinase I isoform α (CK1α). In this complex, β-catenin is phosphorylated at the N-terminal domain (first at Ser45 by CK1α and then at Ser33, Ser37, and Thr41 by GSK-3β), followed by polyubiquitination and subsequent degradation by the ubiquitin-proteasome-mediated pathway (13, 14).

In intercellular adhesion, β-catenin localizes to the plasma membrane, acting as a bridge between E-cadherin and cytoskeleton-associated actin to form adherent junctions between cells (13). BFT is a metalloprotease and can destroy the tight junctions in the intestinal epithelium by cleaving E-cadherin, resulting in the release of β-catenin and the loss of tight junctions (2, 15, 16). From the perspective of clinical findings associated with ETBF infection, these results may lead to the leakage of the intestinal barrier and the diarrhea that are characteristically observed in ETBF infection (15, 16). However, the role of β-catenin as a cellular signaling intermediate in the induction of proinflammatory responses by BFT has not been clarified.

NF-κB is a dimeric transcription factor composed of homodimers or heterodimers of Rel proteins, of which there are five family members in mammalian cells (i.e., RelA [p65], c-Rel, Rel B, NF-κB1 [p50], and NF-κB2 [p52]) (6, 17). We previously demonstrated that BFT primarily induces p65 and p50 heterodimers in intestinal epithelial cells (6, 18). These NF-κB dimers are held in the cytoplasm in an inactive state by physical interaction with IκB proteins. Therefore, IκBα is a negative regulator of NF-κB signaling. In the context of enteric inflammation, the regulation of β-catenin and IκBα seems to be similar. Thus, β-catenin and IκBα are associated with phosphorylation of the same N-terminal serine sequence sites, ubiquitination by the same E3 ligase complex, and subsequent proteasomal degradation (19). As evidence to support this, the degradation of β-catenin has been related to the activation of NF-κB signals in intestinal epithelial cells infected with *Salmonella* (20, 21). However, the opposite results have also been reported. For example, inflammatory cytokine expression and NF-κB activity are all significantly reduced when β-catenin is knocked down in human monocytic THP-1 cells stimulated with the Der p1 allergen derived from the house dust mite (22) and bronchial epithelial BEAS-2B cells stimulated with lipopolysaccharide (LPS) (23). In addition, it has been proposed that β-catenin could influence AP-1 activation in intestinal epithelial cells. For example, a recent report demonstrated that canonical Wnt signaling collaborated with or antagonized the AP-1 transcription factor to fine-tune the expression of shared target genes in the colorectal epithelium (24). Moreover, the loss of membrane-associated E-cadherin after BFT treatment of human colonic epithelial cells triggers the nuclear localization of β-catenin (25). Therefore, it is possible that β-catenin may regulate the BFT-induced activation of transcription factors, such as NF-κB and AP-1. However, there are no reports regarding the role of β-catenin in BFT-induced NF-κB and AP-1 activation in intestinal epithelial cells.

In the studies reported here, we investigated the role of β-catenin in modulating the proinflammatory responses mediated by NF-κB or AP-1 subsequent to BFT exposure in intestinal epithelial cells and found that β-catenin expression was associated with downregulated NF-κB activity after BFT stimulation, leading to the regulation of inflammatory responses, such as IL-8 expression.

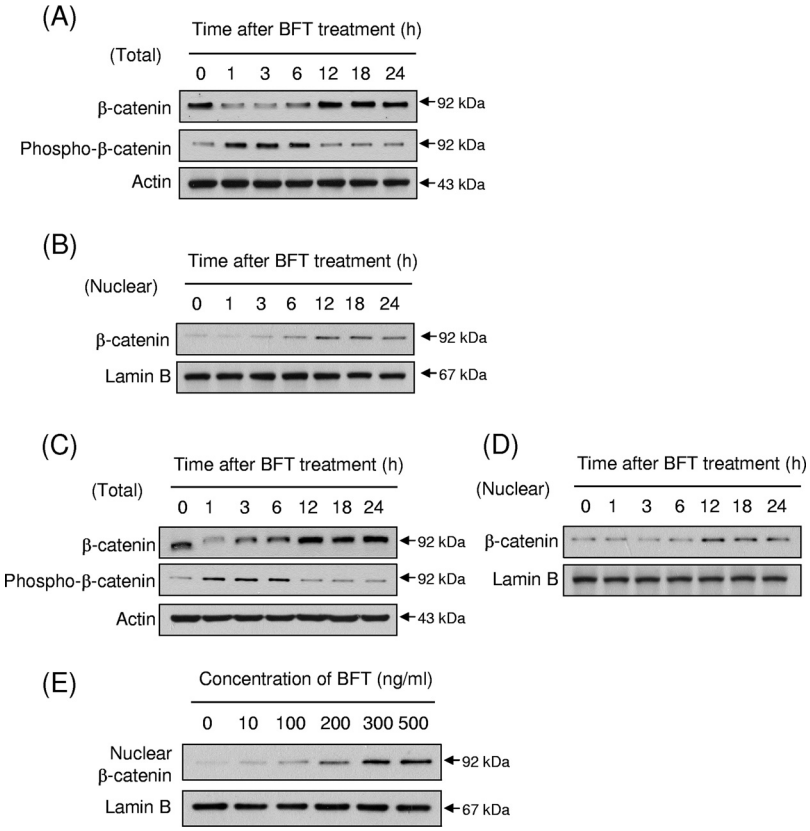

FIG 1 β-Catenin expression in intestinal epithelial cells stimulated with BFT. (A) HCT 116 cells were treated with BFT (300 ng/ml) for the indicated periods of time. The total cellular proteins β-catenin, phospho-β-catenin, and actin were analyzed by immunoblotting. (B) The β-catenin and lamin B in the nuclear fraction were analyzed by immunoblotting. (C and D) Immunoblots for β-catenin levels in CCD 841 CoN cells under the same treatment conditions used for the assays for which the results are shown in panels A and B are shown beneath each time point. (E) HCT 116 cells were treated with each concentration of BFT for 18 h, after which nuclear fractions were obtained. Nuclear β-catenin and lamin B were analyzed by immunoblotting. All results are representative of those from more than three independent experiments.

RESULTS

BFT alters β-catenin expression in intestinal epithelial cells, depending on the time of stimulation. Stimulation of HCT 116 cells with BFT resulted in altered expression of β-catenin. As shown in Fig. 1, early during stimulation β-catenin expression was lower in stimulated cells than in the unstimulated control cells and markedly recovered by 12 h after treatment with BFT, as assessed by immunoblotting (Fig. 1A). In contrast, the levels of phospho-β-catenin were completely different from those of β-catenin. In the nuclear fraction, β-catenin expression increased approximately 12 h after treatment with BFT (Fig. 1B). Similar results were also observed in CCD 841 CoN cells (Fig. 1C and D). Expression of β-catenin translocated into the nucleus was dependent on the concentration of BFT used (Fig. 1E). Based on this result, 300 ng/ml of BFT was used in subsequent experiments.

***β*-Catenin expression leads to downregulation of NF-κB activity in intestinal epithelial cells stimulated with BFT.** β-Catenin is reported to increase or decrease NF-κB activation in various cells (19, 20, 22). As shown in Fig. 2A, HCT 116 cells decreased IκBα expression early after stimulation (at 1 to 6 h poststimulation), and the levels recovered later (at ~12 h poststimulation). Concurrently, expression of phospho-IκBα increased early after stimulation (at 1 to 6 h poststimulation) and then decreased to the baseline thereafter. In addition, the levels of nuclear phospho-p65 increased similarly to the patterns of phospho-IκBα expression. These results suggest that BFT can activate NF-κB signals in HCT 116 cells. Accordingly, we asked whether NF-κB

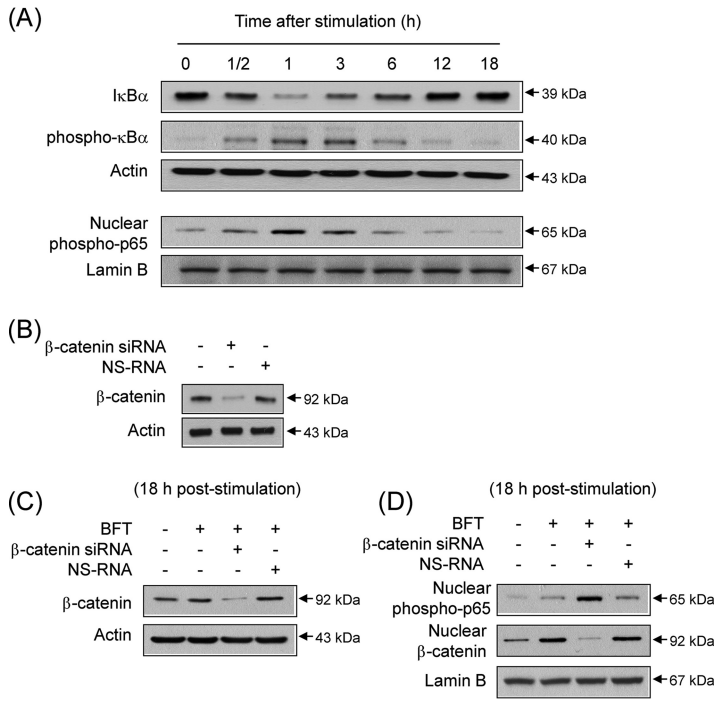

FIG 2 Effects of β-catenin on NF-κB activation in intestinal epithelial cells stimulated with BFT. (A) HCT 116 cells were treated with BFT (300 ng/ml) for the indicated periods of time. (Top) The expression of IκBα, phospho-IκBα, and actin in whole-cell lysates was analyzed by immunoblotting. (Bottom) Immunoblotting results for phospho-p65 and lamin B in nuclear extracts tested under the same conditions used for the assays for which the results are provided in the top panels. (B) HCT 116 cells were transfected with β-catenin-specific siRNA or nonsilencing control siRNA (NS-RNA) as a control. After 48 h, the expression of β-catenin and actin was analyzed by immunoblotting. The results are representative of those from three independent experiments. (C) The transfected cells were stimulated with BFT (300 ng/ml) for 18 h, and the expression of β-catenin and actin was analyzed by immunoblotting. (D) The transfected cells were stimulated with BFT (300 ng/ml) for 18 h. The expression of phospho-p65, β-catenin, and lamin B in the nuclear fractions was analyzed by immunoblotting. The results shown are representative of those from more than three independent experiments. (E) Transfected and untransfected cells were treated with BFT (300 ng/ml) for 18 h. The activities of NF-κB were measured by an ELISA kit. The data are expressed as the mean fold induction ± SEM relative to that for the untreated controls ($n = 5$). *, $P < 0.05$. (F) Transfected and untransfected cells were treated with BFT (300 ng/ml) for the indicated periods of time. The levels of IL-8 were analyzed by quantitative RT-PCR. Values are expressed as the mean ± SD ($n = 5$). *, $P < 0.05$ compared to the results for BFT-treated untransfected cells. In this experimental system, the β-actin mRNA levels in each group remained relatively constant throughout the same periods (~10^6 transcripts/μg total RNA). (G) Transfected and untransfected cells were treated with BFT (300 ng/ml) for 24 h. The amount of IL-8 in the culture supernatants was measured by an ELISA kit. The data are expressed as the mean ± SEM ($n = 5$). *, $P < 0.05$. (H) Transfected and untransfected cells were treated with BFT (300 ng/ml) for 1 h. The activities of NF-κB were measured by an ELISA kit. The data are expressed as the mean fold induction ± SEM relative to that for the untreated controls ($n = 5$). NS, statistically nonsignificant; *, $P < 0.05$.

activation by BFT stimulation may be associated with altered expression of β-catenin in intestinal epithelial cells. For these experiments, transfection with small interfering RNA (siRNA) against β-catenin was used. The period of stimulation of HCT 116 cells with BFT was set to 18 h because the nuclear translocation of β-catenin was observed relatively late after stimulation (at 12 to 24 h poststimulation). As shown in Fig. 2B, transfection with β-catenin siRNA apparently suppressed β-catenin expression in unstimulated cells. In addition, β-catenin siRNA clearly suppressed β-catenin expression under BFT-stimulated conditions (Fig. 2C). Under this experimental condition, the nuclear phospho-p65 signals in BFT-stimulated cells were enhanced compared with those in untransfected cells (Fig. 2D). Concurrently, β-catenin translocated into the nucleus in BFT-stimulated cells was attenuated compared with that in untransfected cells.

To confirm these results, transfected and untransfected cells were stimulated with BFT for 18 h and NF-κB activities were then assessed by enzyme-linked immunosorbent

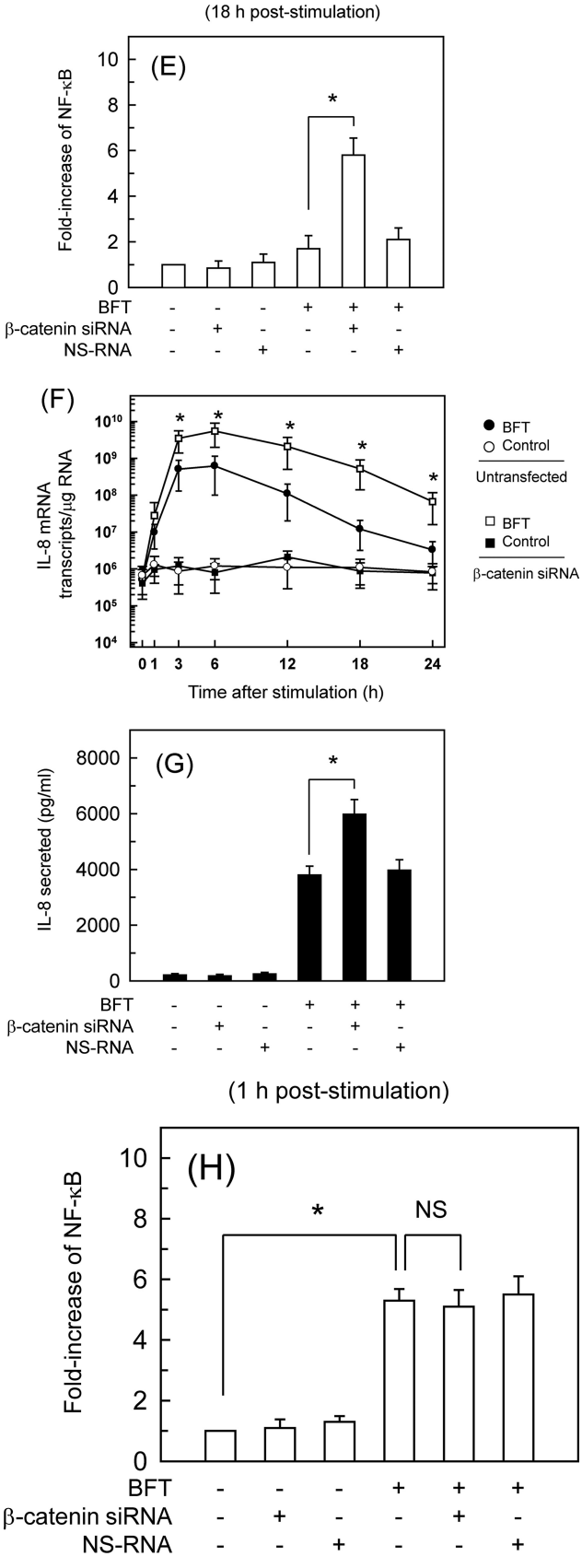

FIG 2 (Continued)

assay (ELISA). NF-κB activities in β-catenin siRNA-transfected cells were significantly higher than those in untransfected cells under BFT-stimulated conditions (Fig. 2E). In the next experiment, IL-8 mRNA expression was measured. Stimulation of untransfected cells with BFT increased IL-8 mRNA expression, with a significant increase in IL-8 mRNA expression first being noted 1 h after treatment with BFT. The expression peaked 3 to 6 h after stimulation and decreased, as assessed by quantitative reverse transcriptase PCR (RT-PCR). In this experimental system, suppression of β-catenin expression significantly enhanced IL-8 mRNA expression compared with that in untransfected cells under BFT-stimulated conditions (Fig. 2F). To determine whether increased IL-8 mRNA levels were accompanied by increased protein secretion, we measured the amount of IL-8 protein in the culture supernatants. As shown in Fig. 2G, the level of IL-8 secretion induced by BFT stimulation rose significantly when β-catenin expression was suppressed using β-catenin siRNA.

We next performed an experiment for observing the effects of β-catenin siRNA at shorter time points prior to β-catenin translocation. For this experiment, the duration of stimulation of HCT 116 cells with BFT was set to 1 h because the nuclear translocation of β-catenin was not observed 1 h after BFT treatment. The results showed that suppression of β-catenin did not significantly change the BFT-induced NF-κB activation (Fig. 2H). Consistent with this, no significant difference in IL-8 mRNA expression was observed between β-catenin siRNA-transfected and untranfected cells 1 h after treatment with BFT (Fig. 2F). These results suggest that the β-catenin-induced suppression of NF-κB and IL-8 signaling may depend on the nuclear translocation of β-catenin in BFT-exposed cells.

In the next experiment, we asked whether overexpression of β-catenin could influence the activities of NF-κB in intestinal epithelial cells stimulated with BFT. For these experiments, HCT 116 cells were transfected with lentiviruses containing a β-catenin-overexpressing plasmid (Fig. 3A). In this experimental model, the period of stimulation of HCT 116 cells with BFT was set to 1 h because the decreased expression of β-catenin was clearly observed in the relatively early period of stimulation. As shown in Fig. 3B, the nuclear levels of phospho-p65 in β-catenin-overexpressing cells were markedly lower than those in untransfected cells under BFT-stimulated conditions. Consistent with this observation, β-catenin overexpression significantly affected BFT-induced NF-κB activities (Fig. 3C). The levels of both IL-8 mRNA expression (Fig. 3D) and IL-8 production (Fig. 3E) in β-catenin-overexpressing cells were also significantly lower than those in untransfected cells under BFT-stimulated conditions.

To confirm these results, CCD 841 CoN cells were pretreated with the β-catenin inhibitor *N*-(6-methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-3-(2-methoxyphenyl)-4-oxothieno[3,2-*d*]pyrimidin-2-yl)thio]-acetamide (IWP-4), and NF-κB activity was then measured by ELISA. As shown in Fig. 4A, treatment with IWP-4 significantly changed the levels of NF-κB activity, showing that inhibition of β-catenin resulted in the prolonged activation of NF-κB. In addition, IL-8 secretion was significantly enhanced by the combined treatment of cells with IWP-4 and BFT compared with that in BFT-treated cells (Fig. 4B). These results suggest a negative regulation of NF-κB activation by β-catenin in BFT-exposed intestinal epithelial cells.

β-Catenin did not influence phospho-IKK activation in intestinal epithelial cells stimulated with BFT. Since NF-κB activation is regulated by IκB kinase α/β (IKKα/β) signals in BFT-exposed epithelial cells (18, 26, 27), we assessed whether β-catenin expression may influence IKK signaling. For this experiment, HCT 116 cells were transfected with siRNA against β-catenin and then stimulated with BFT for 1 h. Stimulation of HCT 116 cells with BFT increased the levels of phosphorylated IKKα/β, in which transfection with β-catenin siRNA did not affect the levels of phosphorylated IKKα/β compared with those in untransfected cells under BFT-exposed conditions (Fig. 5A). In addition, overexpression of β-catenin did not change the expression of phosphorylated IKKα/β (Fig. 5B).

To confirm these results, the activity of phosphorylated IKK was measured by a phospho-IκBα kinase assay kit. There was no significant difference in phospho-IκBα

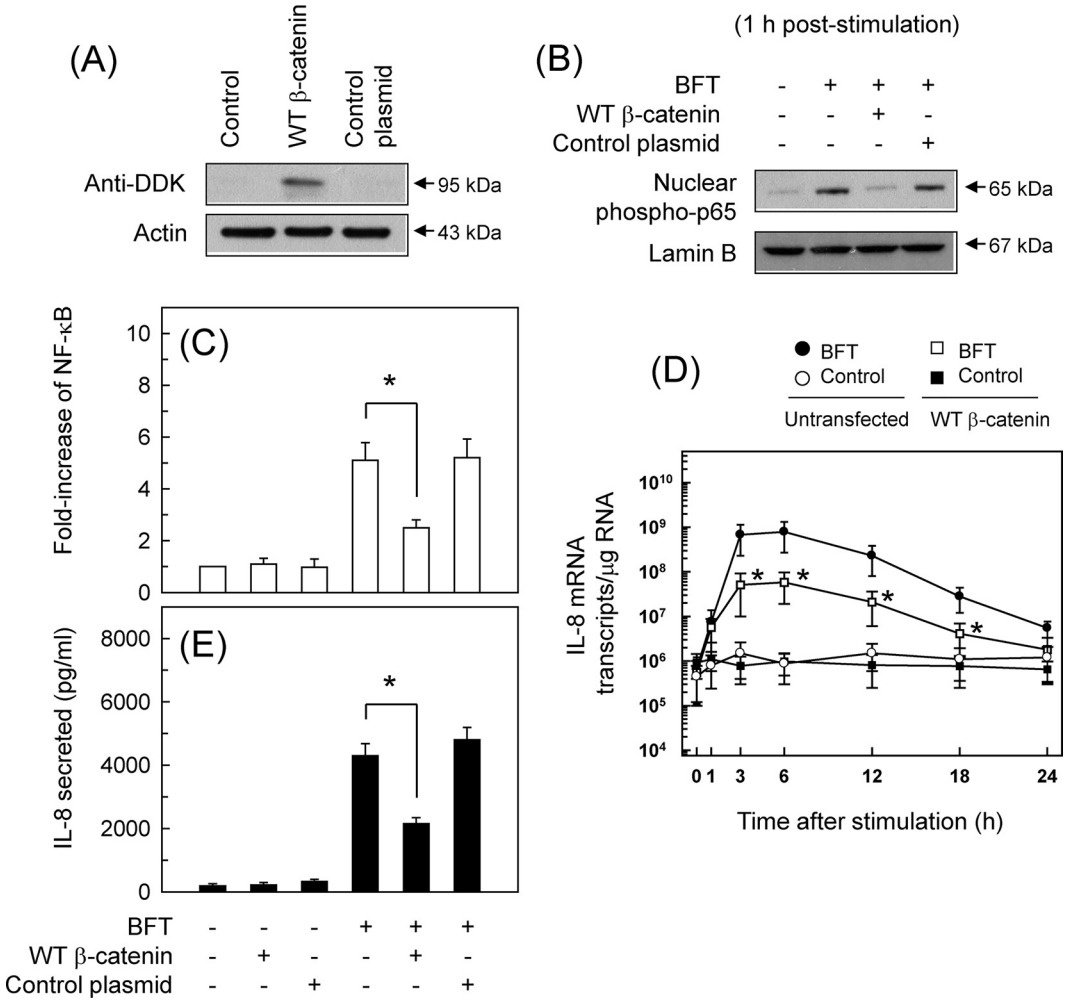

FIG 3 Effects of β-catenin overexpression on NF-κB activation and IL-8 expression in intestinal epithelial cells stimulated with BFT. (A) HCT 116 cells were transfected with lentivirus containing wild-type (WT) β-catenin-overexpressing or control plasmids. The expression of β-catenin proteins was analyzed by immunoblotting using an anti-DDK Ab. The results are representative of those from three independent experiments. (B) WT β-catenin-overexpressing cells were treated with BFT (300 ng/ml) for 1 h. The expression of phospho-p65 signals in the nuclear factions was assessed by immunoblotting. The results are representative of those from three independent experiments. (C) β-Catenin-overexpressing cells were stimulated with BFT (300 ng/ml) for 1 h. The activities of NF-κB were measured by an ELISA kit. The data are expressed as the mean fold induction ± SEM relative to that for the untreated controls ($n = 5$). *, $P < 0.05$. (D) Transfected and untransfected cells were treated with BFT (300 ng/ml) for the indicated periods of time. The levels of IL-8 were analyzed by quantitative RT-PCR. Values are expressed as the mean ± SD ($n = 5$). *, $P < 0.05$ compared to the results for BFT-treated untransfected cells. The β-actin mRNA levels in each group remained relatively constant throughout the same periods (~10^6 transcripts/μg total RNA). (E) β-Catenin-overexpressing cells were stimulated with BFT (300 ng/ml) for 24 h. The amount of IL-8 in the culture supernatants was measured by an ELISA kit. The data are expressed as the mean ± SEM ($n = 5$). *, $P < 0.05$.

activity between β-catenin siRNA-transfected and untransfected cells treated with BFT (Fig. 5C). In the next experiment, CCD 841 CoN cells were used. As shown in Fig. 5D, phospho-IκBα activity was higher in BFT-exposed β-catenin siRNA-transfected CCD 841 CoN cells than in untransfected cells treated with BFT. In this experimental system, pretreatment with IWP-4 did not significantly change phospho-IκBα activity under BFT-stimulated conditions.

***β*-Catenin did not influence AP-1 activation in intestinal epithelial cells stimulated with BFT.** Since it has been reported that canonical Wnt signaling collaborates with or antagonizes the AP-1 transcription factor to fine-tune the expression of shared target genes in the colorectal epithelium (24), we assessed whether β-catenin could regulate AP-1 activation in BFT-exposed cells. Stimulation of HCT 116 cells with BFT activated phospho-c-Jun signals (Fig. 6A). In this experimental system, cells were

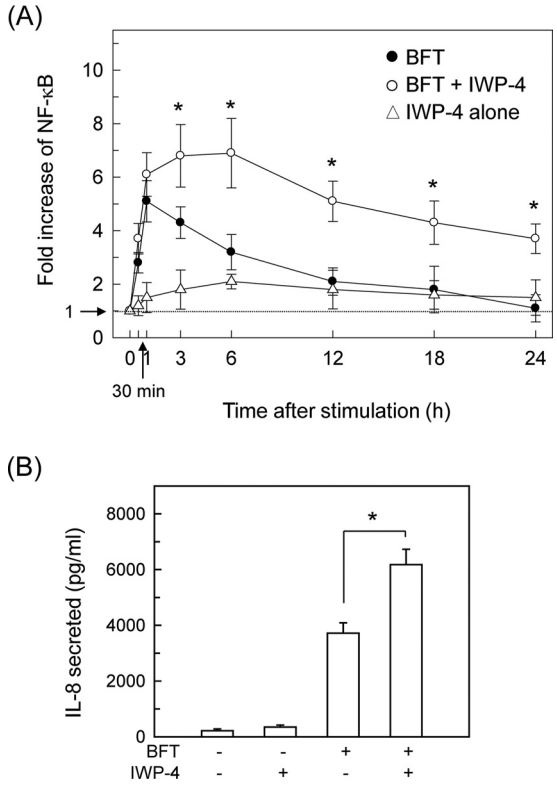

FIG 4 Suppression of β-catenin enhances NF-κB activity and the secretion of IL-8 in intestinal epithelial cells stimulated with BFT. (A) CCD 841 CoN cells were pretreated with the β-catenin inhibitor IWP-4 (100 nM) for 30 min, after which the cells were stimulated with BFT (300 ng/ml) for the indicated periods of time. The activities of NF-κB were measured by an ELISA kit. The data are expressed as the mean fold induction ± SEM relative to that for the untreated controls ($n = 5$). *, $P < 0.05$ in the group treated with BFT and IWP-4 combined compared with the group treated with BFT alone. (B) Cells were pretreated with the β-catenin inhibitor IWP-4 (100 nM) for 30 min, after which cells were stimulated with BFT (300 ng/ml) for 24 h. The amounts of IL-8 in the culture supernatant were measured by an ELISA kit. The data are expressed as the mean ± SEM ($n = 5$). *, $P < 0.05$.

transfected with β-catenin-specific siRNA and the levels of AP-1 activity were measured by ELISA. As shown in Fig. 6B, there was no significant change in the increased AP-1 activity in β-catenin siRNA-transfected cells from that in untransfected cells under BFT-stimulated conditions. To confirm these results, CCD 841 CoN cells were pretreated with the β-catenin inhibitor IWP-4 and then stimulated with BFT for 1 h or 18 h. The level of AP-1 activity following combined treatment with IWP-4 and BFT was not significantly different from that in cells treated with BFT alone for 1 h (Fig. 6C) and 18 h (Fig. 6D).

GSK-3β regulates β-catenin-dependent NF-κB activation and IL-8 expression in intestinal epithelial cells stimulated with BFT. Since GSK-3β, a constitutively active serine-threonine kinase, is known to be a negative regulator of β-catenin (19), we examined whether GSK-3β signaling might influence β-catenin-dependent NF-κB activation and IL-8 expression in intestinal epithelial cells stimulated with BFT. For this experiment, siRNA against GSK-3β was used. As shown in Fig. 7A, transfection with GSK-3β siRNA apparently suppressed GSK-3β expression in unstimulated cells. In addition, GSK-3β siRNA almost suppressed the GSK-3β signal under BFT-stimulated conditions (Fig. 7B). In this experimental model, cells transfected with GSK-3β siRNA had increased levels of IκBα and β-catenin compared with untransfected cells under BFT-exposed conditions. In contrast, the levels of nuclear phospho-p65 expression were markedly reduced in GSK-3β siRNA-transfected cells compared with untransfected cells stimulated with BFT (Fig. 7C).

In the next experiment, the levels of β-catenin and NF-κB were measured in the presence or absence of GSK-3β siRNA. As shown in Fig. 7D, the total cellular levels of

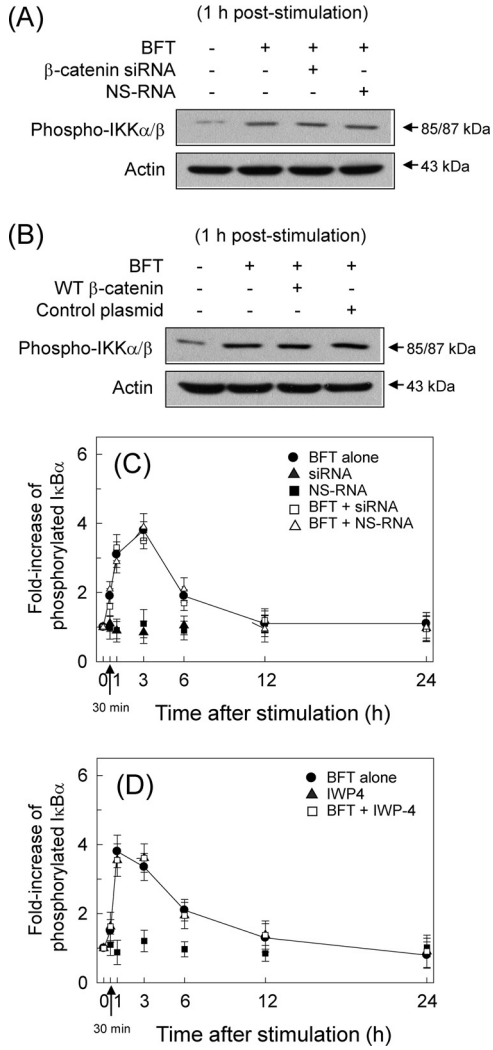

FIG 5 Relationship between β-catenin and IKK activation in intestinal epithelial cells stimulated with BFT. (A) HCT 116 cells were transfected with β-catenin-specific siRNA or nonsilencing siRNA (NS-RNA) as a control for 48 h. The transfected cells were combined with BFT (300 ng/ml) for 1 h, and the expression of phospho-IKKα/β was analyzed by immunoblotting. The results shown are representative of those from more than three independent experiments. (B) HCT 116 cells were transfected with lentivirus containing wild-type (WT) β-catenin-overexpressing plasmids. Transfected cells were stimulated with BFT (300 ng/ml) for 1 h, and the phospho-IKKα/β protein was then analyzed by immunoblotting. The results are representative of those from three independent experiments. (C) HCT 116 cells transfected with β-catenin siRNA or NS-RNA were stimulated with BFT (300 ng/ml) for the indicated periods of time. IKK activity was measured by a phospho-IκBα kinase assay kit. The data are expressed as the mean fold induction ± SEM of phosphorylated IκBα relative to that for the untreated controls ($n = 5$). (D) CCD 841 CoN cells were pretreated with the β-catenin inhibitor IWP-4 (100 nM) for 30 min, after which the cells were stimulated with BFT (300 ng/ml) for the indicated periods of time. IKK activities were measured by a phospho-IκBα kinase assay kit. The data are expressed as the mean fold induction ± SEM of phosphorylated IκBα relative to that for the untreated controls ($n = 5$).

β-catenin were lower in BFT-exposed cells than in the untreated controls. In this experimental system, β-catenin levels were higher in GSK-3β siRNA-transfected cells than in untransfected cells under BFT-stimulated conditions. In contrast, the levels of NF-κB activity were lower in GSK-3β siRNA-transfected cells than in untransfected cells under BFT-stimulated conditions (Fig. 7E).

Next, the levels of IL-8 mRNA were determined by quantitative RT-PCR. As shown in Fig. 7E, transfection with GSK-3β siRNA significantly suppressed IL-8 mRNA expression compared with that in untransfected cells under BFT-exposed conditions (Fig. 7F). In this experimental system, β-actin mRNA levels in each group remained relatively

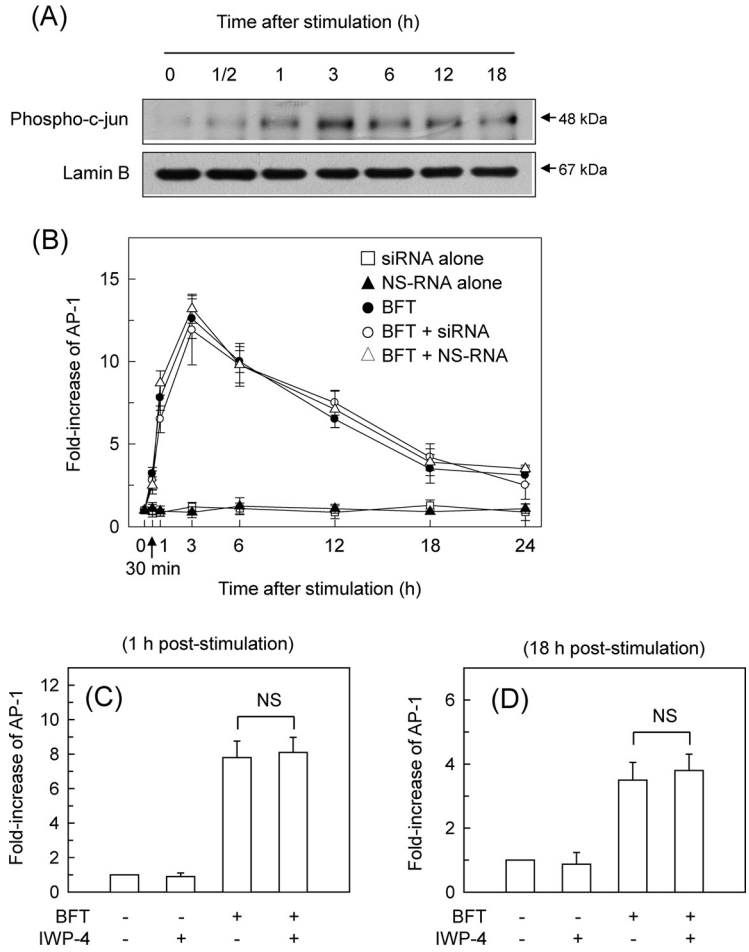

FIG 6 Effects of β-catenin suppression on AP-1 activation in intestinal epithelial cells stimulated with BFT. (A) HCT 116 cells were treated with BFT (300 ng/ml) for the indicated periods of time. The expression of phospho-c-Jun and lamin B in nuclear extracts was analyzed by immunoblotting. The results shown are representative of those from more than three independent experiments. (B) HCT 116 cells were transfected with β-catenin-specific siRNA or nonsilencing siRNA (NS-RNA) as a control for 48 h. Transfected and untransfected cells were treated with BFT (300 ng/ml) for the indicated periods of time. The activities of AP-1 were measured by an ELISA kit. The data are expressed as the mean fold induction ± SEM relative to that for the untreated controls ($n = 5$). (C and D) CCD 841 CoN cells were pretreated with the β-catenin inhibitor IWP-4 (100 nM) for 30 min, after which the cells were stimulated with BFT (300 ng/ml) for 1 h (C) and 18 h (D). The activities of AP-1 were measured by an ELISA kit. The data are expressed as the mean fold induction ± SEM relative to that for the untreated controls ($n = 5$). NS, statistically nonsignificant.

constant throughout the same periods (~10^6 transcripts/μg total RNA). Consistent with this, the levels of IL-8 protein were lower in GSK-3β siRNA-transfected cells than in untransfected cells under BFT-treated conditions (Fig. 7G). To confirm these results, CCD 841 CoN cells were pretreated with LiCl, an inhibitor of GSK-3β, for 24 h and then stimulated with BFT. In this experimental system, the levels of both NF-κB activity and IL-8 secretion were lower with combined treatment with LiCl and BFT than with BFT treatment alone (Fig. 8A and B). These results indicate that suppression of GSK-3β may lead to an increase in β-catenin levels and a reduction of NF-κB activity and IL-8 expression in BFT-exposed intestinal epithelial cells.

DISCUSSION

The virulence factor BFT, produced by the noninvasive bacterium ETBF, initially contacts and stimulates intestinal epithelial cells. The intestinal epithelial cells exposed to BFT then cause enteritis by expressing proinflammatory mediators, such as IL-8, through NF-κB activation (5, 6, 28). NF-κB activation in BFT-exposed cells increases early

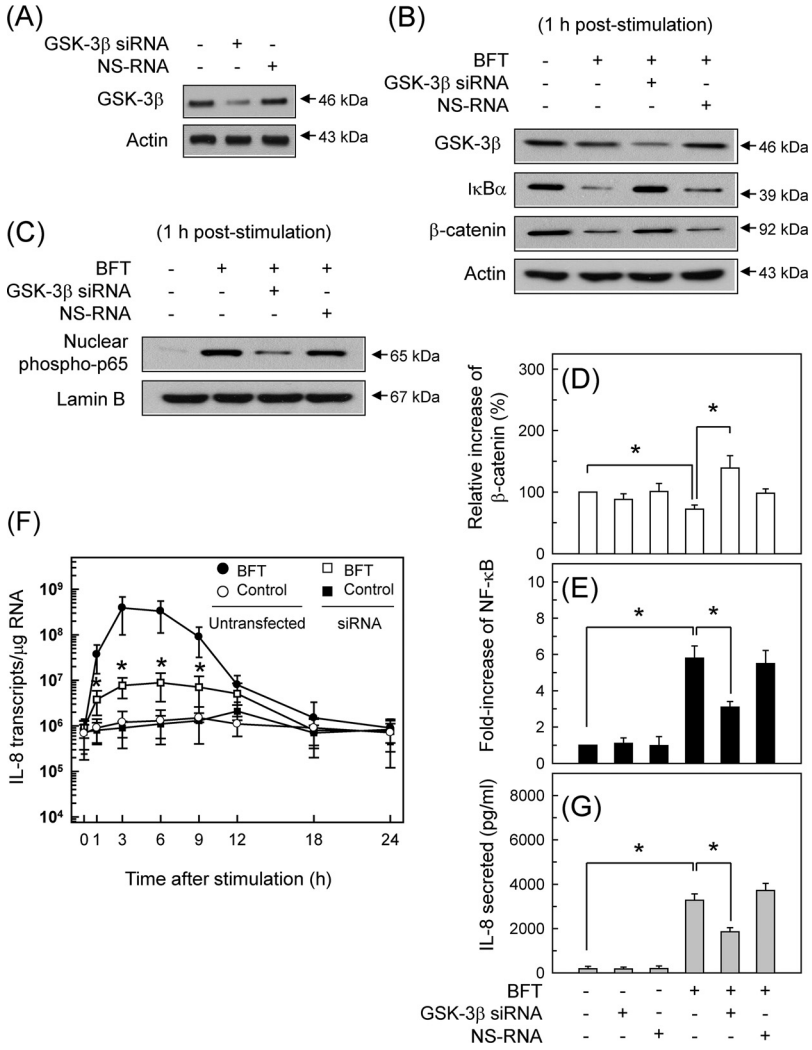

FIG 7 Effects of GSK-3β inhibition on β-catenin expression, NF-κB activation, and IL-8 expression in intestinal epithelial cells stimulated with BFT. (A) HCT 116 cells were transfected with GSK-3β-specific siRNA or nonsilencing siRNA (NS-RNA) as a control for 48 h. The expression of GSK-3β and actin was analyzed by immunoblotting. The results are representative of those from three independent experiments. (B) The transfected cells were combined with BFT (300 ng/ml) for 1 h, after which GSK-3β, IκBα, β-catenin, and β-actin were analyzed by immunoblotting. The results shown are representative of those from more than three independent experiments. (C) Transfected and untransfected cells were treated with BFT (300 ng/ml) for 1 h. The phospho-p65 and lamin B signals in the nuclear fractions were examined by immunoblotting. The results shown are representative of those from more than three independent experiments. (D and E) Transfected and untransfected cells were treated with BFT (300 ng/ml) for 1 h. The levels of β-catenin (D) and NF-κB (E) were analyzed by the use of the respective ELISA kits. The data are expressed as the mean percent induction ± SEM relative to that for the untreated controls ($n = 5$, β-catenin) and the mean fold induction ± SEM relative to that for the untreated controls ($n = 5$, NF-κB). *, $P < 0.05$. (F) Transfected HCT 116 cells were treated with BFT (300 ng/ml) for the indicated periods of time. The levels of IL-8 mRNA were analyzed by quantitative RT-PCR. Values are expressed as the mean ± SD ($n = 5$). The β-actin mRNA levels in each group remained relatively constant throughout the same periods (~10^6 transcripts/μg total RNA). *, $P < 0.05$ compared with untransfected cells treated with BFT. (G) Transfected and untransfected cells were treated with BFT (300 ng/ml) for 18 h. The amount of IL-8 in the culture supernatants was measured by an ELISA kit. The data are expressed as the mean ± SEM ($n = 5$). *, $P < 0.05$.

after stimulation and decreases over time (8). Therefore, several regulatory factors appear to act on BFT-induced inflammatory responses. In the present study, we demonstrated that one of the responses to stimulation with BFT was negative regulation of NF-κB activation and IL-8 expression by β-catenin in intestinal epithelial cells.

In the present study, expression of β-catenin in BFT-treated cells was lower relatively early after stimulation and then recovered to normal levels later, with the kinetics being

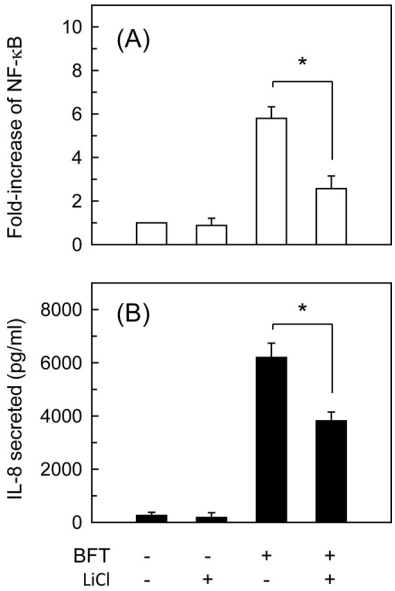

FIG 8 Effects of GSK-3β inhibition on NF-κB activation and IL-8 expression in CCD 841 CoN cells stimulated with BFT. CCD 841 CoN cells were pretreated with LiCl (30 nM) for 24 h, after which the cells were stimulated with BFT (300 ng/ml) for 1 h (NF-κB) or 24 h (IL-8). (A) The activity of NF-κB was measured by an ELISA kit. The data are expressed as the mean fold induction $\pm$ SEM relative to that for the untreated controls ($n = 5$). (B) The amount of IL-8 in culture supernatants was measured by an ELISA kit. The data are expressed as the mean $\pm$ SEM ($n = 5$). *, $P < 0.05$.

similar to those of IκBα in BFT-exposed intestinal epithelial cells. The phosphorylation of β-catenin was higher in the period early after stimulation than in the period late after stimulation. Since β-catenin phosphorylation is known to lead to subsequent proteasomal degradation (29), it seems plausible that the total cellular level of β-catenin would be lower relatively early after stimulation with BFT. Consistent with this, the nuclear translocation of β-catenin was observed later after stimulation. In addition, the nuclear translocation of β-catenin was noticed to coincide with a relatively high level of total cellular β-catenin. Therefore, the nuclear translocation seems to occur only when the total cellular β-catenin level is relatively high. Considering that the high level of translocated β-catenin was related to the inhibition of both NF-κB activity and IL-8 expression, the nuclear levels of β-catenin, which are dependent on relatively high levels of intracellular β-catenin, may affect proinflammatory responses to BFT stimulation.

IL-8 expression is regulated by several factors, such as IKK, NF-κB, and AP-1 signals in intestinal epithelial cells stimulated with BFT. For example, we previously demonstrated that IL-8 expression by NF-κB activation is dependent on IKK signaling in BFT-exposed epithelial cells (6). In addition, inhibition of IKKε results in β-catenin activation (30). A pathway including mitogen-activated protein kinase (MAPK) and subsequent AP-1 activation is required for IL-8 expression in intestinal epithelial cells exposed to BFT (7). However, the present study showed that suppression or enhancement of β-catenin expression was not significantly associated with changes in phosphorylated IKKα/β activities in BFT-exposed cells. In addition, suppression of β-catenin did not affect AP-1 activity in response to BFT stimulation. Based on these results, β-catenin appears to determine IL-8 expression by regulating NF-κB activation in BFT-exposed intestinal epithelial cells. Therefore, the stabilization of β-catenin may have an anti-inflammatory effect by attenuating NF-κB-mediated proinflammatory activity, leading to controlling intestinal inflammation in ETBF infection. Further studies are required to clarify if β-catenin modifies intestinal inflammation during the course of *in vivo* infection with ETBF.

In the present study, we used 300 ng/ml of BFT for observing the change of

β-catenin expression in HCT 116 cells (a human colonic epithelial cancer cell line) and CCD 841 CoN cells (a normal colonic epithelial cell line), and similar results were obtained in both cell lines. However, 100 ng/ml of BFT was used in other studies, such as with IL-8 in HT-29 cells (a human colorectal epithelial cell line) (5, 6), lipocalin-2 in T84 cells (a human colorectal epithelial cell line) (8), and HO-1 in CMT-93 cells (a murine intestinal epithelial cell line) (18). In addition, Wu et al. demonstrated that BFT could induce the nuclear localization of β-catenin at 100 ng/ml of BFT in HT29/C1 cells (a clone from the HT29 cell line) (25). Therefore, the reason for the different expression of proinflammatory mediators depending on the concentration of BFT used may be due to differences in experimental cell lines.

BFT, a metalloprotease protein toxin, is reported to cleave the extracellular domain of E-cadherin (15), and its cleavage then releases β-catenin associated with the cytoplasmic domain of E-cadherin at 100 ng/ml of BFT in HT29/C1 cells (25). Therefore, we inferred that 300 ng/ml of BFT seems to be enough for the cleavage of E-cadherin in the HCT 116 and CCD 841 CoN cells used in this study. The loss of E-cadherin at 300 ng/ml of BFT may lead to the disruption of its linkages with β-catenin and further promote colonic permeability and the access of innate mucosal immune cells to luminal bacterial antigens (2, 3). Further research based on the concentration of BFT and *in vivo* experiments will be required.

Given that the loss of membrane-associated E-cadherin after BFT treatment of human colonic epithelial cells triggers the nuclear localization of β-catenin (15, 25, 31), BFT-induced β-catenin in the cytoplasm seems to originate from the complex of E-cadherin linked with β-catenin. The E-cadherin intracellular domain links with the actin cytoskeleton through adhesion molecules, such as β-catenin and α-catenin. In addition, a second source of β-catenin involved in signaling has been reported (25). Thus, the normal degradation of a complex including APC, axin, and GSK-3β can increase the level of cytoplasmic β-catenin (14, 25). We posit that these two sources may help bring cytoplasmic β-catenin back to normal levels in the late response to BFT stimulation.

In the present study, the total cellular expression of GSK-3β was measured in an intestinal epithelial cell model transfected with GSK-3β siRNA. The suppression of GSK-3β resulted in increased expression of β-catenin but a decrease of both NF-κB activity and IL-8 expression in BFT-stimulated cells. Given that the status of GSK-3β activity depends on the site of phosphorylation of GSK-3β (19, 32), further studies are required to clarify the phosphorylated status of GSK-3β in BFT-stimulated cells.

Since the clinical illnesses caused by ETBF infection are typically self-limited with colitis (3, 4), it is plausible that anti-inflammatory factors may be induced during the infection. In the present study, we propose that one of these factors is the β-catenin-induced negative regulation. That is, suppression of β-catenin using siRNA and chemicals resulted in greater NF-κB activity and IL-8 expression after BFT treatment, while overexpression of β-catenin reduced both. Besides IL-8, NF-κB can regulate a variety of proinflammatory mediators, including CXCL1 (6), CCL2 (6), human β-defensin 2 (27), cyclooxygenase 2 (33), and heme oxygenase 1 (18), in BFT-exposed cells. Therefore, translocated β-catenin may regulate these mediators in acute ETBF infection. However, this study was performed exclusively in colonic epithelial cell lines. Therefore, further studies using an *in vivo* model are required to clarify our findings.

The present study demonstrated the negative regulation of NF-κB-mediated inflammatory responses by β-catenin from the point of view of acute inflammation. Nevertheless, different perspectives may be suggested for persons with long-term carriage of ETBF strains. For example, a paper showed that the BFT-induced degradation of E-cadherin augmented β-catenin nuclear signaling with the induction of expression of the oncogene c-Myc, which contributed to the BFT-induced proliferation of colonic epithelial cells (25). Therefore, the long-term carriage of ETBF strains may contribute to a chronic pathology, such as inflammation and oncogenic transformation in the intestine (2–4).

Based on these results, there appears to be a signaling pathway that includes

GSK-3β involved in suppressing β-catenin, NF-κB activation, and IL-8 expression in BFT-exposed intestinal epithelial cells. Consequently, our findings support the hypothesis that β-catenin is a regulating factor that attenuates the colonic inflammatory response caused by BFT.

MATERIALS AND METHODS

Reagents. Antibiotics, the TRIzol reagent, and Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) were obtained from Gibco BRL (Gaithersburg, MD, USA). *N*-(6-Methyl-2-benzothiazolyl)-2-[(3,4,6,7-tetrahydro-3-(2-methoxyphenyl)-4-oxothieno[3,2-*d*]pyrimidin-2-yl)thio]-acetamide (IWP-4), lithium chloride (LiCl), and sodium pyruvate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). LPS-free fetal bovine serum (FBS), McCoy's 5a medium, and Eagle's minimum essential medium (EMEM) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Rabbit monoclonal antibodies (MAbs) against IκBα, phospho-IκBα, phospho-IKKα/β, β-catenin, and GSK-3β and rabbit polyclonal antibodies (Abs) against phospho-p65, phospho-c-Jun, and phospho-β-catenin (Ser33/Ser37/Thr41) were acquired from Cell Signaling Technology, Inc. (Beverly, MA, USA). Mouse MAbs against actin and lamin B and goat anti-mouse and anti-rabbit immunoglobulin secondary Abs conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Purification of BFT and cell culture conditions. BFT was purified from culture supernatants of a toxigenic strain of ETBF (ATCC 43858) as described previously (18, 26, 34). The purity of the BFT preparations was verified, as they appeared as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The activity of LPS in BFT solutions (1 mg/ml) was less than 1 endotoxin unit/ml (Pyrosate test kit; quantitative chromogenic *Limulus* amebocyte lysate; Associates of Cape Cod, Inc., East Falmouth, MA, USA). Using a HEK-Blue LPS detection kit (InvivoGen, San Diego, CA, USA) with a detection limit of 3 ng/ml, the amount of LPS in the BFT solutions (1 mg/ml) was found to be less than 3 ng/ml. BFT was frozen in aliquots at -80°C immediately after purification.

The human colon epithelial cell line HCT 116 (a colorectal carcinoma cell line; ATCC CCL-247) was grown in McCoy's 5a medium with 10% FBS and antibiotics (100 units/ml of penicillin and 100 μg/ml of streptomycin). CCD 841 CoN cells (a colonic epithelial cell line derived from normal tissue; ATCC CRL-1790) were cultured in EMEM supplemented with 10% FBS and 1 mM sodium pyruvate. The cells were seeded at 0.5×10^6 to 2×10^6 cells per well onto six-well plates and allowed to attach overnight. After 12 h of serum starvation, the cells were incubated with BFT.

Transfection. β-catenin siRNA (human; catalog number sc-29209), GSK-3β siRNA (human; catalog number sc-35527), and negative-control (nonsilencing) siRNA (NS-RNA; catalog number sc-37007) were purchased from Santa Cruz Biotechnology. Briefly, cells were cultured in 6-well plates to 50 to 80% confluence and then transfected with siRNA using the Lipofectamine RNAiMax reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Transfected cells were incubated for 48 h prior to the assay.

A lentiviral packing kit and a human β-catenin cDNA clone for lentiviral vectors were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The β-catenin cDNA clone contained DDK (the peptide DYKDDDDK; FLAG tag), and β-catenin expression was verified using an anti-DDK MAb (IgG2a; catalog number TA50011-100; OriGene Technologies, Inc.). Transfection experiments were performed according to the manufacturer's instructions (18). Because there is a level of β-catenin in the cytoplasm under unstimulated conditions (Fig. 1A and C), the difference in β-catenin expression between the control group and the overexpressing plasmid-transfected group may be not properly identified in Western blotting assays using β-catenin antibody. Therefore, we used an anti-DDK MAb instead of the β-catenin antibody.

Quantitative reverse transcriptase PCR (RT-PCR) and ELISA. Cells were treated with BFT, and then total cellular RNA was extracted using the TRIzol reagent. To quantify the expressed mRNA molecules, reverse transcription and PCR amplification for IL-8 and β-actin were performed using an internal standard as described previously (5, 35). PCR amplification consisted of 35 cycles of 1 min of denaturation at 95°C with 2.5 min of annealing and extension at either 60°C (IL-8) or 72°C (β-actin).

The protein levels of IL-8 in the culture supernatant were evaluated using a commercially available kit (R&D Systems, Inc., Minneapolis, MN, USA). The protein levels of β-catenin were also measured with an ELISA kit (Cell Signaling Technology). ELISA kits for the Trans^{AM} NF-κB and AP-1 families were obtained from Active Motif (Carlsbad, CA, USA) (8). A PathScan phospho-IκBα kinase assay kit (Cell Signaling Technology) was used to quantify the amount of phosphorylated IKK (21). Each assay was performed according to the individual manufacturer's instructions.

Immunoblotting. Cells were washed with ice-cold phosphate-buffered saline and lysed in 0.5 ml/well lysis buffer (150 mM NaCl, 20 mM Tris, pH 7.5, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin). Fifteen to 50 μg of protein per lane was size fractionated on a polyacrylamide minigel (Mini-Protein II apparatus; Bio-Rad) and electrophoretically transferred to a nitrocellulose membrane (0.1-μm pore size). Immunoreactive proteins to which primary Abs bound were visualized using goat anti-rabbit or anti-mouse immunoglobulin secondary Abs conjugated to horseradish peroxidase, followed by enhanced chemiluminescence (ECL system; Amersham Life Science, Buckinghamshire, UK) and exposure to X-ray film.

Statistical analyses. Data from quantitative RT-PCR assays are presented as the mean $\pm$ standard deviation (SD). Data from the ELISAs are presented as the mean $\pm$ standard error of the mean (SEM). The Mann-Whitney U test was used for statistical analysis. *P* values of <0.05 were considered statistically significant.

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