Rebamipide suppresses TLR-TBK1 signaling pathway resulting in regulating IRF3/7 and IFN-α/β reduction

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Original Article

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The pathogenesis of inflammatory bowel disease (IBD) remains unclear. The immune response to viral or bacterial infection is thought to be one etiology of intestinal inflammation. Toll-like receptors (TLR) induce innate immune responses by recognizing invading microbial pathogens that cause the activation of adaptive immune responses. 1,2 In TLRs, TLR3 and TLR4 impart ligand-specific recognition of double-stranded RNA (dsRNA) of viruses and of bacterial lipopolysaccharide (LPS), respectively. 3,4 The LPS- or polynosinic-polycytidylate (poly(I:C))-induced activation of the TLR/IL-1R domain-containing adaptor inducing interferon (IFN)-β (TRIF; TICAM-1), 5,6,7 which is an adaptor that functions independently of MyD88, leads to the delayed activation of nuclear factor-κB (NF-κB). 8,9 TRIF also induces activation of the transcriptional regulator, IFN regulatory factor (IRF) 3, and the expression of IFN-β and of IFN-inducible genes through the activation of TANK-binding kinase 1 (TBK1) and inhibitor of kappaB kinase ε (IKKe). 10,11 TLR3 activates primarily the TRIF pathway, whereas TLR4 activates both MyD88- and TRIF-dependent pathways—Both IKKe 12 and TBK1 13,14 are key regulators of the IRF3 and IFN7 activation pathways in cells that have been exposed to viruses and/or activated by dsRNA via TLR3. 15,16 NF-κB is induced by poly(I:C)/LPS through TLR3/4. 15,17 NF-κB activation is required for the release of proinflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF). 18

The initiation and perpetuation of the inflammatory intestinal responses in IBD might result from an exaggerated host defense reaction of the intestinal epithelium to endogenous luminal bacterial flora and viruses via TLR3/4-signaling. TLR3 is significantly down-regulated in intestinal epithelial cells from patients with active Crohn’s disease (CD) but not in those with ulcerative colitis (UC). In contrast, TLR4 is obviously up-regulated in both UC and CD. 19 Moreover, a relationship has been identified between IBD and NF-κB, 20 but not between IBD and the TBK1-IRF pathway.

Rebamipide is widely used in Japan to treat gastric ulcers 21 and gastric injury. 22 Recently, there was a report of administered rebamipide enemas to a patient with IBD complicated by perforation of IBD. 23 The therapeutic efficacy of rebamipide has been independently confirmed by others using a model of colitis induced by acetic acid 24 or dextran sulfate-sodium (DSS). 25,26 However, the relationship between rebamipide and TBK1 has not been described.

Here, we analyzed the relationship between IBD (in particular, UC) and the TLR-TBK1-IRF3/7 pathway. We then evaluated the effect of rebamipide on the TBK1-IRF3/7-IFN-α/β pathway and on the NF-κB activation pathway using colonic epithelial cells and mice with colitis induced by DSS.

Material and Methods

Human samples and tissue collection. Biopsy specimens of normal and moderately or severely inflammatory mucosa obtained during colonoscopy of 10 patients with UC at Nagoya City University Hospital between 2005 and 2007 were stored at −80°C for mRNA detection.

The Ethics Committee of Nagoya City University Graduate School of Medical Sciences granted approval for this study and written informed consent was obtained from all patients to participate in all procedures associated with the study.

Immunohistochemistry of UC patients. Immunohistochemical staining of colon tissues was performed with antibodies

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against TBK1 (EP611Y, abcam, Tokyo, Japan; dilution 1:50). The procedure was performed with the appropriate positive and negative controls. Briefly, 3-µm-thick sections were deparaffinized and hydrated through a graded series of alcohols. After inhibition of endogenous peroxidase activity by immersion in 3% H2O2/methanol solution, antigen retrieval was achieved by heating the samples in 10 mM citrate buffer (pH 6.0) using a microwave oven for 10 min at 98°C. Then, sections were incubated with the primary antibody. After thorough washing in PBS, the samples were incubated with biotinylated the secondary antibody and then with avidin-biotin horseradish peroxidase complexes (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA). Finally, immune complexes were visualized by incubation with 0.01% H2O2 and 0.05% 3,3’-diaminobenzidine tetrachloride (DAB).

Experimental procedures of DSS mice. Eight-week-old female C57BL/6 mice were housed under conventional conditions in a temperature-controlled room with a 12 h light/dark cycle. Colitis was induced in the mice by orally administering 3% dextran sulfate sodium (DSS; MP Biomedicals Inc., Morgan, CA) in distilled water daily for 5 days. The mice were assigned to groups as follows: Control (no DSS and no rebamipide), DSS (oral DSS alone), DSS + rebamipide (oral DSS plus daily rectal administration of 50 mg/kg/day of rebamipide dissolved in 100 µl of 0.5% carboxymethylcellulose (CMC; Wako Pure Chemical Industries Ltd., Osaka, Japan). All mice were sacrificed 5 days after DSS administration was started. The Animal Care Committee of Nagoya City University approved the study protocol.

Histopathology of DSS mice colon tissues. All specimens were routinely processed and stained with hematoxylin and eosin for histological examination.

Cell culture. Human colonic cancer cells (CaCo2; ATCC number, HTB-37) were seeded in 6-cm dishes at a density of 2 × 10^6/dish and cultured for 48 h with RPMI1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum. Cells were disrupted using a Bio-Ruptor sonicator (Cosmo Bio, Tokyo, Japan) for 15 s, and then lysates were centrifuged at 15,000 rpm for 10 min at 4°C. All samples were normalized to an equal protein concentration using a BCA protein assay kit (Bio-Rad Laboratories, CA). An equal quantity of 2× SDS-PAGE sample buffer (0.5 mol/l Tris-HCl (pH 7.2), 150 mol/l NaCl, 1 mol/l Na2EDTA) was loaded to the membranes, and subsequently dissolved in 1× lysis buffer (Cell Signaling Technology) containing 20 mol/l Tris-HCl (pH 7.5), 150 mol/l NaCl, 1 mol/l Na2EDTA, 1 mol/l EGTA, 1% Triton, 2.5 mol/l sodium pyrophosphate, 1 mol/l β-glycerophosphate, 1 mol/l Na3VO4, and 1 µg/ml leupeptin. Cells were disrupted using a Bio-Ruptor sonicator (Cosmo Bio, Tokyo, Japan) for 15 s, and then lysates were centrifuged at 15,000 rpm for 10 min at 4°C. All samples were normalized to an equal protein concentration using a Bio-Rad Laboratories, CA). An equal quantity of 2× SDS-PAGE sample buffer (0.5 mol/l Tris-HCl (pH 7.2), 1% SDS, 100 mol/l β-mercaptoethanol, and 0.01% bromophenol blue) was added to the samples, and then the mixtures were boiled for 5 min at 100°C. Portions of boiled samples were fractioned on 7.5%, 10%, or 12.5% SDS-PAGE gels and then transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). Non-specific binding on the membranes was blocked with 5% skimmed milk in PBS (–) for 1 h at room temperature. The membranes were incubated with TBK1 monoclonal antibody (clone: 108A429, Gene Tex, Inc., Irvine, CA) overnight at 4°C
followed by three washes with 0.05% Tween 20 in PBS (−) at 5-min intervals. The membranes were incubated with an appropriate secondary antibody for 1 h at room temperature, followed by three washes with 0.05% Tween 20 in PBS (−) at 5-min intervals. Immunoreactive proteins were visualized using the ECL Plus Western blotting Detection system (Amersham Biosciences). Filters were stripped and reprobed using monoclonal β-actin antibody (Abcam Plc., Cambridge, England) as an internal control.

**Immunofluorescence microscopy.** CaCo2 cells in a subconfluent state were incubated with Poly(I:C) or LPS at 37°C for 24 h, respectively, in the presence or absence of rebamipide. Thereafter TBK1 was analyzed by immunofluorescence study. Cells were fixed with ethanol and acetone. Incubation with primary antibody of TBK1 was performed in a solution of PBS containing 0.1% milk at room temperature. Then, sections were incubated with the appropriate secondary antibody and all sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Kirkegaard and Perry Laboratories). Images were obtained with an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan).

**Results**

**Inflammatory mucosa of UC over-expressed TBK1-IRF3/7 signaling pathway.** We first performed real-time RT-PCR of TBK1, NAP1, IRF3, and IRF7 to confirm the relationship between the mRNA expression of these genes and UC in 10 patients. The mRNA expression of all these genes was higher in atypical, than in normal mucosa from the patients (Fig. 1). These results indicated that the inflammation associated with UC is related to the TBK1-IRF3/7 signaling pathway.

**Immunohistochemical analysis of TBK1 in UC.** We performed immunohistochemical staining of TBK1 in UC tissues from humans. TBK1 was mainly expressed in obviously inflammatory colon epithelial cells of crypts (Fig. 2 A and B). On the other hand, TBK1 was hardly expressed in colon epithelial cells with weak inflammation (Fig. 2 C and D).

**Histological findings in the colons of DDS mice.** Crypts were diffusely absent and considerable numbers of inflammatory cells had infiltrated colon specimens from mice in the DSS group (Fig. 3B), whereas few crypts had disappeared and inflammatory cell infiltration was minimal in the DSS + rebamipide group (Fig. 3C).

**Rebamipide suppressed TBK1-IRF3/7-IFN-α/β signaling pathway in DDS mice.** To determine the effect of rebamipide on the TLR-TBK1 signaling pathway in colitis, we performed real-time RT-PCR of TBK1, IRF3, IRF7, IFN-α and IFN-β on colon specimens from DSS and from DSS + rebamipide groups. The mRNA expression of all these genes was increased due to inflammation in the colon of DSS mice, whereas such elevation was suppressed in that of the DSS + rebamipide group (Fig. 4). These results indicated that rebamipide suppresses the TBK1-IRF3/7-IFN-α/β signaling pathway in DDS mice.

**Rebamipide suppressed TLR-TBK1 signaling pathway in human colonic epithelial cells.** To clarify the mechanism of rebamipide on the TLR-TBK1 signaling pathway in colitis, we performed both real-time RT-PCR of TBK1, NAP1, IRF3 and IRF7, and Western blotting of TBK1 using human colonic epithelial cells. Poly(I:C) (a TLR3 ligand) was added to CaCo2 colonic epithelial cells with or without rebamipide. Poly(I:C) alone increased the mRNA expression of all of these genes in

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**Fig. 1.** Inflammatory mucosa of UC patients over-expressed TBK1-IRF3/7 signaling pathway in human biopsy tissues. Real-time RT-PCR of TBK1, NAP1, IRF3, and IRF7 confirmed the relationship between mRNA expression of these genes and ulcerative colitis in 10 patients. More TBK1, NAP1, IRF3, and IRF7 mRNA was expressed in atypical, than in the normal mucosa of patients with UC.

**Fig. 2.** Immunohistochemical analysis of TBK1 in UC. TBK1 was mainly expressed in inflammatory colon epithelial cells of crypts (A), but hardly expressed in colon epithelial cells with weak inflammation (C). Higher magnification of A (B). Higher magnification of C (D). (Original magnification: A, C ×200; B, D ×400).

**Fig. 3.** Histological findings in the colon of DDS mice. Normal colon mucosa of untreated mouse (A). Colon specimen from DSS mouse shows diffuse crypt disappearance and inflammatory cell infiltration (B) indicating severe colitis. Colon specimen from mouse given oral DSS and daily rectal rebamipide, shows minimal crypt disappearance and inflammatory cell infiltration (C).
the cells. However, the mRNA expression of these genes was suppressed to control levels when both poly(I:C) and rebamipide were added to the cells (Fig. 5). LPS, which is a ligand for TLR4, increased the mRNA expression of all these genes in CaCo2 cells, whereas rebamipide suppressed these LPS-induced increases to control levels (Fig. 6). Western blotting showed that rebamipide suppressed TBK1 protein expression (Fig. 7). Immunofluorescence image of TBK1 indicated that rebamipide with LPS suppressed TBK1 protein expression (Fig. 8). Rebamipide also suppressed the expression of TBK-1 with poly(I:C) (data not shown). These results indicated that rebamipide suppresses the TLR3/4-TBK1 signaling pathway in...
human colonic epithelial and serves as an important factor in anti-inflammation via this pathway. To determine the effect of rebamipide on the NF-κB signaling pathway, we performed real-time RT-PCR of NF-κB in human colonic epithelial cells. LPS alone increased, whereas LPS plus rebamipide suppressed mRNA expression of all of these genes to control levels in CaCo2 cells.

**Fig. 6.** Rebamipide suppressed TLR4-TBK1 signaling pathway in human colonic epithelial cells. Lipopolysaccharide (TLR4 ligand) was added to CaCo2 colonic epithelial cells with or without rebamipide. LPS alone increased, whereas LPS plus rebamipide suppressed mRNA expression of all of these genes to control levels in CaCo2 cells.

**Fig. 7.** Effect of rebamipide on protein expression level of TBK1. Western blots show that rebamipide suppressed TBK1 protein expression in human colonic epithelial cells induced by either poly(I:C) or LPS. These results indicate that rebamipide suppresses TLR3/4-TBK1 signaling in these cells.

**Fig. 8.** Immunofluorescent image of TBK1. TBK1 expression was visualized by immunofluorescent microscopy. Nuclei were stained blue by DAPI. TBK1 was clearly expressed in the cytoplasm of CaCo2 cells treated with LPS (A). On the other hand, TBK1 expression was reduced in the cytoplasm of CaCo2 cells treated with LPS and rebamipide (C).

**Fig. 9.** Effect of rebamipide on NF-κB signaling pathway. Real-time RT-PCR of NF-κB in human colonic epithelial cells shows that LPS and poly(I:C) each increased NF-κB gene mRNA expression in colonic epithelial cells, and that adding rebamipide did not suppress either of these increases.

human colonic epithelial and serves as an important factor in anti-inflammation via this pathway. To determine the effect of rebamipide on the NF-κB signaling pathway, we performed real-time RT-PCR of NF-κB in CaCo2 cells using the same procedure as described above. We found that LPS increased the expression of NF-κB mRNA in CaCo2 cells (Fig. 9), whereas LPS/poly(I:C) plus rebamipide did not suppress the expression and the level was similar to that in the presence of LPS/poly(I:C) (Fig. 9). These results indicated that rebamipide does not exert anti-inflammatory effects via the NF-κB signaling pathway.
Discussion

The TLRs are the best-characterized family of innate immune receptors and they recognize conserved microbial motifs including dsRNA and LPS. Several intestinal cell types including epithelial cells, dendritic cells, monocyte/macrophages, granulocytes and lymphocytes express TLRs. Studies using mice deficient in the MyD88 gene, which is the major signaling adaptor of the TLR family, suggest that the predominant role of TLR/MyD88 signal transduction is to prevent intestinal inflammation. However, TLR/MyD88 signaling also promotes intestinal inflammation, and the inhibition of NF-κB, a major target of this pathway, can ameliorate murine colitis. Rebamipide also has mucosal protective and healing actions.

In conclusion, the TLR-TBK1-IRF3/7 pathway might be one etiology of IBD and rebamipide directly thwarts this pathway. However, further studies are required to clarify the mechanism of action.

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