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CUTTING EDGE

Cutting Edge: The IκB Kinase (IKK) Inhibitor, NEMO-Binding Domain Peptide, Blocks Inflammatory Injury in Murine Colitis[1]

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***Inflammatory mediators such as TNF-α, IL-6, and IL-1 are important in the pathogenesis of inflammatory bowel diseases and are regulated by the activation of NF-κB. The aim of the present study was to investigate whether the NF-κB essential modulator (NEMO)-binding domain (NBD) peptide, which has been shown to block the association of NEMO with the IκB kinase β subunit (IKKβ) and inhibit NF-κB activity, reduces inflammatory injury in mice with colitis. Two colitis models were established by the following: 1) inclusion of dextran sulfate sodium salt (DSS) in the drinking water of the mice; and 2) a trinitrobenzene sulfonic acid enema. Marked NF-κB activation and expression of proinflammatory cytokines were observed in colonic tissues. The NBD peptide ameliorated colonic inflammatory injury through the down-regulation of proinflammatory cytokines mediated by NF-κB inhibition in both models. These results indicate that an IKKβ-targeted NF-κB blockade using the NBD peptide could be an attractive therapeutic approach for inflammatory bowel disease. The Journal of Immunology,* 2007, 179: 2681–2685.**

Inflammatory bowel diseases (IBD)[3] (Crohn's disease and ulcerative colitis) are believed to be caused by a combination of genetic predisposition and environmental factors that affect the response to Ags presented by microflora and the production of inflammatory mediators (1, 2). IBD are associated with the infiltration into the intestinal lamina propria of mononuclear cells such as macrophages or lymphocytes that overexpress the products of NF-κB target genes, including the proinflammatory cytokines TNF-α, IL-1β, and IL-6 (2, 3). Therapy for IBD has recently been enriched by the successful development of anti-cytokine biologic agents such as infliximab (chimeric anti-human TNF-α mAb), adalimumab (recombinant human anti-human TNF-α mAb), and anakinra (recombinant form of human IL-1 receptor antagonist). However, all of the available anti-cytokine biologicals are proteins and suffer to a certain degree from the general disadvantages associated with protein drugs. Therefore, small molecular anti-cytokine agents that can target the specific pathways of proinflammatory cytokines would be attractive candidates for anticytokine biologics. Because aberrant NF-κB activation, probably caused by exposure to enteric bacteria, is thought to be critical to the development of IBD, many of the anti-inflammatory drugs used to treat IBD inhibit NF-κB activation (3, 4).

Numerous stimuli, including proinflammatory cytokines, bacterial pathogens, and even radiation, activate NF-κB, mostly through IκB kinase (IKK)-dependent phosphorylation and degradation of IκB proteins. NF-κB dimers can translocate to the nucleus where they modulate the transcription of genes that encode cytokines, chemokines, and antiapoptotic factors (5, 6). The IKK complex consists of two protein kinase subunits, IKKα and IKKβ, and a regulatory component, NF-κB essential modulator (NEMO)/IKKγ (7). IKK activation by most stimuli requires phosphorylation of the IKKβ subunit at two serine residues (5). IKKβ deficiency results in an increased susceptibility to TNF-α-induced apoptosis and an absence of innate immunity, as well as a deficiency of p65 (8, 9). Thus, selective inhibition of the IKK complex has been proposed as a promising target to block aberrant NF-κB activity in inflammatory diseases such as IBD.

The present study was conducted to investigate the effect of specific inhibition of IKKβ-mediated NF-κB activation on murine colitis using the well-characterized NEMO-binding domain (NBD) peptide (10). We found that the NBD peptide reduced disease symptoms and ameliorated histological pathology in IBD-related mouse models, which suggests a clinical application for IBD treatment.

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; APDC, ammonium pyrrolidine dithiocarbamate; Cox-2, cyclooxygenase-2; DSS, dextran sulfate sodium salt; Et-OH, ethanol; IKK, IκB kinase; NEMO, NF-κB essential modulator; NBD, NEMO-binding domain; mutNBD, mutated NBD; TNBS, trinitrobenzene sulfonic acid; wt-NBD, wild-type NBD.

Materials and Methods

Cell culture and treatment

J774.1 cells were cultured as previously described (10). Confluent cultures were treated with bacterial LPS (from *Escherichia coli*; Sigma-Aldrich) at the indicated time points. The cells and supernatant were collected and used to prepare protein extracts or for ELISA.

Mice

C57BL/6J mice were purchased from Tokyo Laboratory Animals Science (Tokyo, Japan) and maintained under standard laboratory conditions. All of the described experimental protocols were approved by the Ethics Committee for Animal Experimentation and, conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Department of Medicine, University of Tokyo, Tokyo, Japan.

Treatment of mice with NBD peptide or other inhibitors

The NBD peptides were as previously described (11). The wild-type (wtNBD) and mutated (mutNBD) NBD peptides contained the Antennapedia homeodomain (in lower case) and IKK (in upper case) segments as follows with the positions of the W to A mutations underlined: wtNBD, drqikiwfqnrrmkwkk TALDWSWLQTE; and mutNBD, drqikiwfqnrrmkwkkTALDASALQTE. Groups of mice were treated with wtNBD peptide or mutNBD peptide *via* i.p. injection in a blinded fashion at dosages of 0.1 mg/kg (dextran sulfate sodium salt (DSS) model) or 1.0 mg/kg (trinitrobenzene sulfonic acid (TNBS) model) of body weight once a day at the indicated times. Aspirin and ammonium pyrrolidine dithiocarbamate (APDC), which are reported to inhibit NF-κB activation, were also used for the treatment of mice (12, 13). Control mice received injections of PBS as the vehicle.

DSS colitis

The NBD treatment and histological scoring were as follows: mice (6-wk-old males) were given DSS (ICN Biomedicals) in their drinking water for 5 days as indicated and thereafter they were provided regular water. When indicated, mice were also treated with NBD (0.1 mg/kg) in PBS, aspirin (200 mg/kg), or APDC (100 mmol/kg) via i.p. injection once daily from day 1 to day 5. For histologic and gene expression analyses, the mice were killed 10 days after the initiation of DSS treatment. Histologic scoring of fixed (10% formaldehyde) and sectioned (paraffin-embedded) tissues was performed in a blinded manner as described by Dieleman et al. (14) as follows: inflammation (0 (none) to 3 (severe)), extent (0 (none) to 3(transmural)), regeneration (0 (complete regeneration or normal tissue) to 3 (no tissue repair)), crypt damage (0 (none) to 3 (entire crypt and epithelium lost)), and the number of ulcers. The five scores were summed to give a total score. Grading was performed in a blind fashion by an expert pathologist.

TNBS model

Mice were lightly anesthetized with pentobarbital sodium salt and administered a haptenating agent, 2.5 mg TNBS dissolved in 50% ethanol (Et-OH), intrarectally via a 3.5 French catheter equipped with a 1-ml syringe; the catheter was advanced into the rectum until the tip was 4 cm proximal to the anal verge, at which point the haptenating agent was administered in a total volume of 150 μl. To ensure distribution of the haptenating agent within the entire colon and cecum, mice were held in a vertical position for 1 min after the intrarectal injection. Control mice were administered an Et-OH solution without the haptenating agent by using the same technique. Mice were also treated with NBD (1.0 mg/kg) in PBS or with PBS alone via the i.p. route at 4, 24, and 48 h after TNBS administration. Histologic scoring of fixed (10% formaldehyde) and sectioned (paraffin-embedded) tissues was performed in a blinded manner. The scoring system was as follows: 0, normal; 1, mild mucosal inflammation without ulceration; 2, moderate mucosal inflammation without ulceration; 3, severe mucosal inflammation with ulceration (< 2 mm) or no ulceration; and 4, severe mucosal inflammation with ulceration (> 2 mm). Four randomly selected sections were graded and the average value is indicated.

Western blotting

Protein lysates were prepared from cultured macrophages, separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and analyzed by immunoblotting.

EMSA

Protein lysates were prepared from cultured macrophages. Supernatants that contained nuclear extracts were collected and used for EMSA with a ^{32}P-end-labeled, double-stranded NF-κB oligonucleotide (5′-AGTTGAGGGG ACTTTCCCAGGC-3′), according to the manufacturer's instructions (Promega).

RNA extraction, real-time PCR, and ELISA

Total cellular RNA was extracted from the colon tissue using the ISOGEN reagents (Nippon Gene) according to the manufacturer's instructions. The cDNA was generated using SuperScript II (Invitrogen Life Technologies) and the amounts of the different mRNAs were measured by real-time PCR using GAPDH mRNA for normalization. The primer sequences are available upon request. Cytokine levels were measured by ELISA.

Immunohistochemistry

Colons were fixed in 10% formaldehyde, dehydrated, embedded in paraffin, and sectioned (5-μm thickness). Sections were deparaffinized, rehydrated, treated with 3% H_2O_2 in PBS, and incubated overnight at 4°C with anti-F4/80 (Caltag Laboratories), anti-phospho IκBα (Cell Signaling), anti-p65 (Santa Cruz Biotechnology), anti-cyclooxygenase-2 (Cox-2) (Cayman Chemical), anti-IL-6 (R&D Systems), or identical concentrations of isotype-matched control Abs. Binding of the primary Ab was detected with biotin-labeled anti-rabbit IgG or anti-rat IgG Abs (1/500 dilution; Vector Laboratories) followed by a streptavidin-HRP reaction and visualization with 3,3′-diaminobenzidine (Sigma-Aldrich) and counterstaining with hematoxylin.

Statistical analysis

Differences between means were compared by Student's t tests; $p < 0.05$ was considered significant.

Results and Discussion

NBD peptide blocks LPS-induced NF-κB activity and proinflammatory cytokine expression

Initially, we examined the effect of the wtNBD peptide on NF-κB activation by LPS in J774.1 murine macrophages, which is the major cell type that accumulates in murine colitis. IκBα phosphorylation, degradation, p65 phosphorylation, and IKK phosphorylation were observed after 15–30 min of LPS treatment, indicating that the NF-κB signaling pathway had been activated. The wtNBD peptide (10 μM), but not the mutNBD peptide, inhibited the phosphorylation and degradation of IκBα by LPS treatment. NF-κB binding activity was also inhibited by wtNBD in the EMSA analysis (Fig. 1*A*). Because IKKβ/NF-κB deficiency results in increased susceptibility to LPS-induced apoptosis (8), we examined the effect on cell viability of LPS treatment with or without the wtNBD peptide. The wtNBD peptide did not affect cell viability at doses of up to 20 μM. However, 40 μM wtNBD peptide resulted in decreased cell viability (Fig. 1*B*). To explore the effect of wtNBD on LPS-mediated inflammatory cytokine expression in macrophages, we treated J774.1 cells with LPS with or without wtNBD peptide, collected the supernatant after 24 h, and determined the IL-6, IL-1β, and TNF-α concentrations by ELISA. The IL-6, IL-1β, and TNF-α levels were markedly increased, and the wtNBD peptide reduced the concentrations of IL-6, IL-1β, and TNF-α in dose-dependent manners (Fig. 1*C*). We also determined mRNA expressions by real-time PCR. The IL-6, IL-1β, and TNF-α mRNA levels were markedly increased by LPS, and the wtNBD peptide reduced the expressions in dose-dependent manners (Fig. 1*D*).

Effect of NBD peptides on DSS-induced colitis

To determine the function of IKKβ/NF-κB in colitis, mice were given 3% DSS in their drinking water for 5 days with the wtNBD peptide, the mutNBD peptide, or the other NF-κB inhibitors, aspirin and APDC, and were monitored for weight loss, which is a characteristic of severe intestinal inflammation. After 5 days, the body weights of mice without the wtNBD peptide started to decrease until day 10, when they were sacrificed and analyzed; a 14% total decrease in body weight was recorded

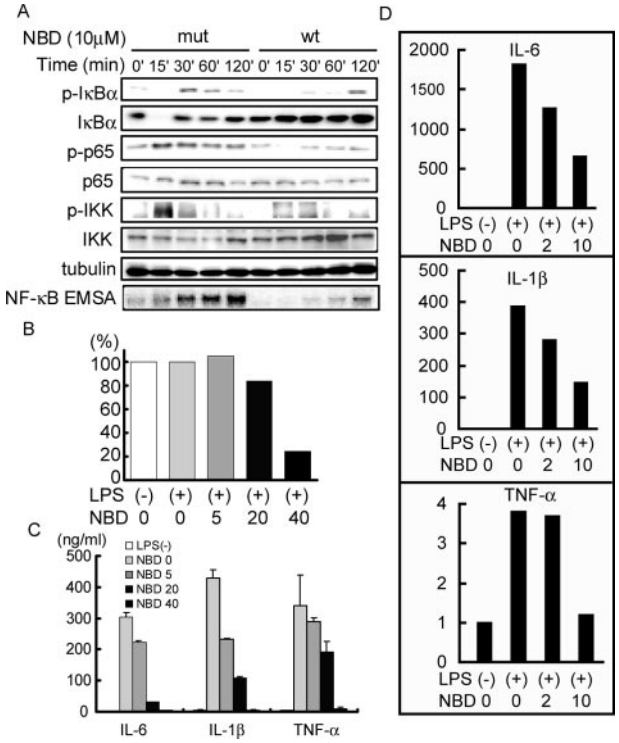

FIGURE 1. The wtNBD peptide specifically blocks LPS-induced NF-κB activity and proinflammatory cytokine expression. *A*, J774.1 macrophages were preincubated with the wtNBD or the mutNBD peptide at a concentration of 10 μM for 30 min. Subsequently, the cells were stimulated with 100 ng/ml LPS for 120 min. IκBα phosphorylation (p-IκBα), degradation, p65 phosphorylation (p-p65), p65, IKK phosphorylation (p-IKK), IKK, and tubulin were determined by immunoblot. NF-κB activation was analyzed by EMSA. *B*, J774.1 cells were incubated with the indicated concentrations of wtNBD peptide and LPS (100 ng/ml). After 24 h, cell viability was analyzed. *C*, J774.1 cells were incubated with the indicated concentrations of wtNBD peptide and LPS (100 ng/ml). Supernatants were harvested after 24 h and secreted cytokines were measured by ELISA. Results are expressed as the means ± SD. The values for IL-6 and IL-1β are magnified five times and 10^3 times, respectively. *D*, J774.1 cells were incubated with the indicated concentrations of wtNBD peptide and LPS (100 ng/ml). RNA was extracted after 4 h, and mRNA expressions were determined by real-time PCR. Results indicate fold increase in normalized mRNA (relative to GAPDH mRNA).

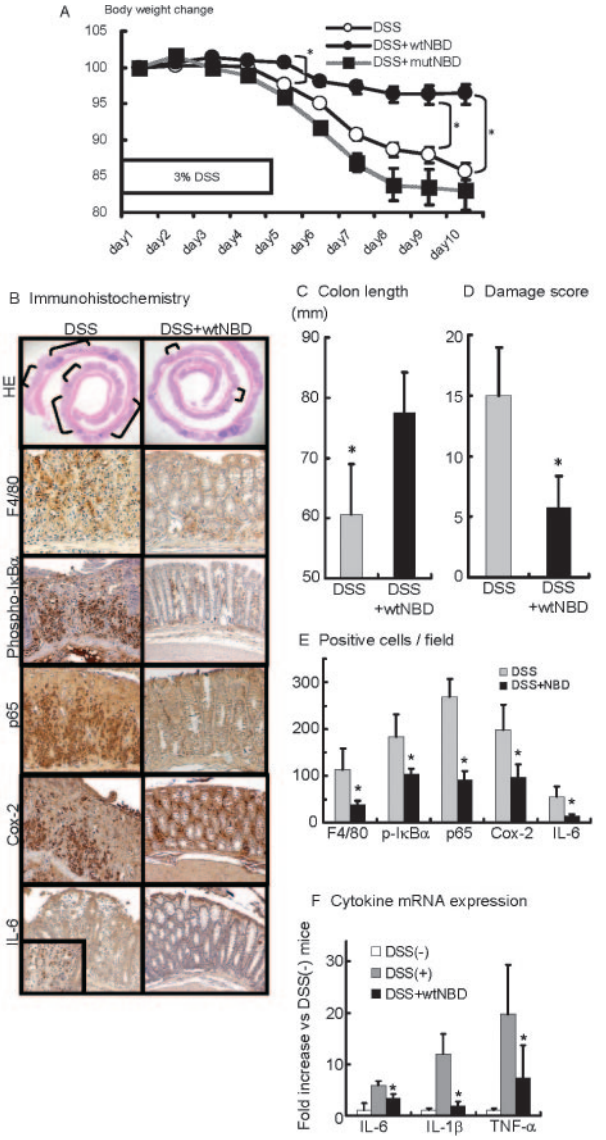

FIGURE 2. Effect of NBD peptide on DSS-induced colitis. *A*, Mice treated with the wtNBD peptide or mutNBD peptide were given 3% DSS in their drinking water for 5 days and weighed daily. Body weight is shown as a percentage of the starting weight. Each NBD peptide was injected via the i.p. route once a day for the first 5 days. Data shown are representative of three independent experiments, mean values ± SE, and derived from 10 mice per group. *B*, Typical histological appearance of the DSS-exposed colon with or without wtNBD peptide 10 days after DSS-induced colitis. HE, H&E; brackets indicate borders of ulcers. Magnification, ×5. Macrophage infiltrates into colonic tissue prepared 10 days after the initiation of DSS exposure were analyzed by indirect immunoperoxidase staining with anti-F4/80 Ab. The expression patterns of phospho-IκBα, p65, Cox-2, and IL-6 in DSS-treated mice with or without the wtNBD peptide were analyzed by immunohistochemistry. *C*, Lengths of the inflamed colons on day 10 after DSS initiation with or without NBD. *, $p < 0.05$, compared with DSS alone. *D*, Histological scores of tissue specimens obtained on day 10 after DSS initiation from mice that received DSS alone or DSS plus wtNBD. *, $p < 0.05$. *E*, Cells stained for phospho-IκBα, p65 nuclear staining, Cox-2, and IL-6 were counted in different areas of the colon 10 days after receiving DSS with or without the wtNBD peptide. *F*, The GAPDH-normalized mRNA levels of IL-6, IL-1β, and TNF-α in colon tissues from the DSS model were determined by real-time PCR analysis. Data are plotted as the mean ± S.E. ($n = 10$/group). An asterisk indicates a statistically significant difference (*, $p < 0.05$) relative to the vehicle control group.

on day 10. In contrast, the body weights of mice with the wtNBD peptide showed only a 5% total decrease on day 8 and thereafter they started to recover (Fig. 2*A*); we did not observe similar increases in the body weights of mice treated with the other two NF-κB inhibitors (data not shown). Histologic analyses revealed that the severity and extent of inflammatory lesions in the colons of wtNBD peptide-treated mice were significantly ($p < 0.05$) lower than in untreated mice and that they had smaller areas of ulceration (Fig. 2, *B* and *D*). Numerous macrophages had infiltrated the DSS-mediated colitis mice as determined by F4/80 immunostaining. After DSS exposure, IKKβ/NF-κB activity determined by phospho-IκBα and nuclear p65 immunostaining was found to have decreased in the colons of wtNBD peptide-treated mice compared with untreated mice (Fig. 2, *B* and *E*). The amounts of Cox-2 and IL-6 protein, which are expressed in colitis, were significantly lower in the colons of wtNBD peptide-treated mice relative to their nontreated counterparts (Fig. 2, *B* and *E*). Phospho-IκBα, nuclear p65, Cox-2, and IL-6 were predominantly expressed in F4/80-positive macrophages within inflammatory lesions (Fig. 2*B*). Shortening of the colon, which is another characteristic of severe inflammation, was significantly inhibited in mice that received the wtNBD peptide (Fig. 2*C*). Mice treated with the

wtNBD peptide expressed lower amounts of mRNAs for the proinflammatory cytokines IL-6, IL-1β, and TNF-α in their colons relative to untreated mice (Fig. 2*F*).

Treatment of TNBS-induced colitis by administration of the NBD peptide

We next examined the effects of the wtNBD and mutNBD peptides in another murine colitis model that involves the administration of TNBS in Et-OH into the mouse rectum. Initially, we monitored the survival of mice and found that mice treated with the wtNBD peptide had a significantly increased survival rate (80%) relative to untreated mice (40%) (Fig. *3A*). Treatment with Et-OH, which was used as the TNBS vehicle, did not result in mortality. Whereas mice that received TNBS exhibited progressive weight loss, which is a characteristic of severe intestinal inflammation, those treated with the wtNBD peptide showed significantly less weight loss and started to recover on day 4 after TNBS administration (Fig. 3*B*). Shortening of the colon, which is another characteristic of severe inflammation, was significantly improved in mice treated with the wtNBD peptide (Fig. 3*C*). Histologic analyses revealed that the severity and extent of inflammatory lesions in the colons of wtNBD peptide-treated mice were significantly ($p < 0.05$) lower than in untreated mice and that ulcerated areas were smaller than those seen in the DSS model (Fig. 3, *D* and *E*). Mice treated with wtNBD peptide expressed lower levels of mRNAs for proinflammatory cytokines in their colons relative to Et-OH-treated mice (Fig. 3*F*). These results indicate that IKKβ/NF-κB activation is one of the critical regulators in TNBS colitis and that blockade by wtNBD peptide may offer a new therapeutic approach for human IBD.

In the present study, we demonstrate that the wtNBD peptide, which is a well-characterized IKK inhibitor, ameliorates colitis-related disease in two well-established mouse models of IBD. Among the common cellular pathways activated in IBD, NF-κB is thought to be the most common pathway central to cell activation and the production of various inflammatory mediators, including a variety of cytokines and chemokines. Thus, inhibition of NF-κB activity is a promising strategy for blocking inflammation. To date, several investigators have reported that the use of a NF-κB decoy with or without viral delivery is very effective for ameliorating murine IBD (15, 16). Compared with other NF-κB inhibitors, the wtNBD peptide has an important advantage in that it works upstream of the master kinase IKK in the cytoplasm. Because NF-κB is a dimer that consists of several NF-κB family members, inhibitors must be designed for several activation domains (17). In contrast, the wtNBD peptide blocks IKK activity, which results in the blockade of most NF-κB activity. Another advantage is that the appropriate dosage of wtNBD peptide does not affect basal NF-κB activity while NF-κB activity that is inducible by proinflammatory stimuli is effectively blocked, indicating that the normal cellular functions of NF-κB are unaffected and that the wtNBD peptide is safer than other NF-κB inhibitors (10). In addition, we show that the wtNBD peptide at the appropriate concentration does not increase cell death but does decrease cytokine expression.

In the development of DSS colitis, activated macrophages may play an important role by producing proinflammatory cytokines such as IL-6 and TNF-α. Treatment with the wtNBD peptide reduced the production of these cytokines in the colonic mucosa as shown by immunohistochemistry in the present study. Thus, the target cells of the wtNBD peptide may be macrophages. Besides proinflammatory cytokine production, the activation of NF-κB is strongly linked to the inhibition of apoptosis due to regulation of the expression of anti-apoptotic genes, such as cellular inhibitors of apoptosis (c-IAPs), Bcl-x, and cellular FLIP (c-FLIP). The finding that mice lacking the RelA subunit of either NF-κB or IKKβ die during mid-gestation due to TNF-α-mediated hepatocyte apoptosis (8, 18) raises serious concerns about the safety of IKK inhibitors. For instance, extensive inhibition of NF-κB activation in the colon may result in the loss of colonic mucosa in patients that experience elevated levels of systemic TNF-α. In fact, cell type-specific deletion of IKKβ in colonic epithelial cells results in severe colitis following DSS treatment (19). Consistent with this result, we observed that overdose of the wtNBD peptide resulted in cell toxicity by apoptosis in vitro and severe colitis in vivo (data not shown). Additionally, in the acute colitis caused

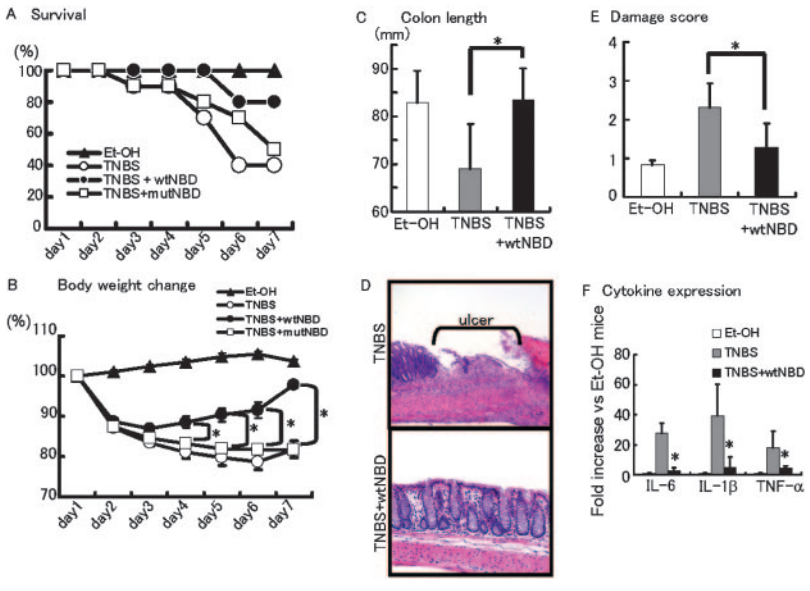

FIGURE 3. Treatment of TNBS-induced colitis by the administration of NBD peptides. Colitis was induced by rectal administration of TNBS on day 1. Mice were treated with a NBD peptide via the i.p. route (at 4, 24, and 48 h). The data shown are representative of three independent experiments. *A*, Survival curves of mice treated with TNBS, TNBS plus wtNBD, TNBS plus mutNBD, or vehicle (Et-OH) alone. *B*, Body weight curves of mice treated with TNBS, TNBS plus wtNBD, TNBS plus mutNBD, or vehicle (Et-OH) alone. The data shown are mean values ± SE for 10 mice per group. *, $p < 0.05$. *C*, Lengths of the inflamed colons of mice treated with either TNBS or TNBS plus wtNBD. *, $p < 0.05$. *D*, Typical histological appearance of the colon on day 10 after TNBS initiation from mice that received TNBS alone or TNBS plus wtNBD. Magnification, ×100. *E*, Histological scores of tissue specimens from mice that received TNBS, TNBS plus wtNBD or vehicle (Et-OH) alone. *, $p < 0.05$. *F*, GAPDH-normalized mRNA levels of IL-6, IL-1β, and TNF-α from the colon tissues of the TNBS model were determined by real-time PCR analysis. The data are plotted as the mean ± S.E. ($n = 10$/group). An asterisk indicates a statistically significant difference (*, $p < 0.05$) compared with the vehicle control group.

by bacterial infection the inhibition of NF-κB resulted in severe colitis because some of the antimicrobial peptides were regulated by NF-κB (20). However, we observed that wtNBD had no effect on the acute colitis caused by *Citrobacter rodentium*, a member of a family of human and animal pathogens that includes the clinically significant enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli* strains (data not shown). Even though this compound may be very effective, it is important to evaluate and monitor IKK inhibitors such as the wtNBD peptide before performing clinical trials.

Many anti-inflammatory drugs for the treatment of IBD inhibit NF-κB activity, at least partially. Of these, glucocorticoids and 5-aminosalicylic acid are reported to be strong inhibitors of NF-κB activity, which is thought to be one of the reasons behind their efficacy (3). We have demonstrated that the wtNBD peptide, which can potently inhibit NF-κB activity, ameliorates murine colitis by preventing the expression of proinflammatory cytokines and may represent a novel therapeutic approach for IBD.

Disclosures

The authors have no financial conflict of interest.

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