Pro-inflammatory signaling by Jun-N-terminal kinase in inflammatory bowel disease

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Abstract. Since Jun-N-terminal kinase participates in intracellular signaling cascades resulting in inflammatory responses, inhibiting this pathway may represent a new treatment for inflammatory bowel disease including ulcerative colitis and Crohn's disease. However, the functional significance of the activation of this kinase in inflammatory bowel disease remains unclear. We investigated whether Jun-N-terminal kinase activation is increased in inflammatory bowel disease and analyzed the effects of SP600125, which decreases inflammatory cytokine synthesis by inhibiting the phosphorylation of this kinase. Phosphorylation of the kinase was examined in affected human colon using an enzyme-linked immunosorbent assay and immunohistochemistry. The effect of SP600125 on cytokine production was examined in cultures of patients' leukocytes and colonic tissue. Finally, rats received injection of SP600125 (30 mg/kg, s.c.) or vehicle twice daily 2 h before the induction of colitis with dextran sulfate sodium. SP600125 effects were determined observationally and histologically. Colonic tissue contained increased phosphorylated kinase in patients with inflammatory bowel disease with expression localized to the nucleus of epithelial and lamina propria mononuclear cells in lesions. Culturing mononuclear cells or colonic tissue with SP600125 down-regulated inflammatory cytokine production. Prophylactic treatment with SP600125 significantly reduced clinical and pathological scores in dextran sulfate sodium-treated rats. This first demonstration of the pathogenetic role of Jun-N-terminal kinase in the development of intestinal inflammation suggests that inhibiting its phosphorylation could benefit patients with inflammatory bowel disease.

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Introduction

Although recent intensive investigations have greatly advanced our understanding of the etiopathogenesis of inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis, the specific causes have yet to be identified. Mechanisms abnormally and persistently activating inflammation leading to ongoing tissue damage are varied and complex, with cytokines playing a central regulatory role (1,2). Recent immunological research has focused on the identification of intracellular signaling pathways and transcription factors through which cytokines act (3,4). Mediators released during IBD activate intracellular signaling cascades regulated by kinases and phosphatases. Mitogen-activated protein kinases (MAPKs) are components of signaling cascades where diverse extracellular stimuli converge to initiate inflammatory cellular responses (5,6). Several subgroups have been identified within the MAPK family, including the p42/44 extracellular signal-related kinase (ERK), Jun-N-terminal kinase (JNK), and p38 MAPK.

JNK has been implicated as an important regulator of the coordinated release of cytokines by immunocompetent cells and of the response of neutrophils to inflammatory stimuli (7,8). Many different stiumuli can activate JNK, including lipopolysaccharide (LPS) and other bacterial products, cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, growth factors, and stresses such as heat shock, hypoxia, and ischemia/reperfusion (9-14). In addition, JNK positively regulates a variety of genes involved in inflammation, such as those encoding TNF-α, IL-1, and IL-6. Although an understanding of the functions of many MAPKs has been facilitated by characterization using specific inhibitors, no specific JNK inhibitor has been available until very recently. In a pilot clinical trial in patients with Crohn's disease, administration of CNI-1493 (15), a synthetic guanylhydrazone that inhibits phosphorylation of both p38 MAPK and JNK, significantly decreased disease activity (16). JNK appeared to be a more relevant CNI-1493 treatment target than p38 MAPK since JNK phosphorylation was inhibited more completely than p38 phosphorylation in LPS-stimulated peripheral blood mononuclear cells (PBMC) *in vitro*, as well as in mucosal inflammatory cells *in vivo*. This implication of JNK was

supported further by the finding that, although the p38 MAPK inhibitor SB203580 effectively inhibited p38 MAPK enzymatic activity in mice with colitis, no attenuation of disease progression was observed (17).

SP600125 has been identified as a specific inhibitor of JNK (18). The *in vivo* effects of SP600125 on inhibition of cytokine synthesis and protection against tissue injury have been evaluated in several different animal models of inflammation including adjuvant-induced arthritis (19) and pulmonary inflammation (20) in rats. Accordingly, pharmacological inhibition of JNK has been advocated as a potential therapeutic strategy for IBD.

The aims of the present study were to determine the colonic expression of activated JNK in IBD, to investigate the effect of a novel selective JNK inhibitor (SP600125) on cytokine production *in vitro* by blood cells and tissue samples from IBD patients, and to use SP600125 to examine whether JNK blockade could suppress colonic inflammation in a rat model of IBD.

Materials and methods

JNK inhibitor. SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) is a novel JNK inhibitor synthesized by the Department of Chemistry at the Signal Research Division of Celgene (San Diego, CA). Its chemical and biochemical properties have been reported elsewhere (18,19).

Studies in human cells and tissue

Patients and samples. PBMC were obtained from normal subjects and patients with active ulcerative colitis and Crohn's disease. Biopsy specimens of inflamed colon were obtained during colonoscopy. Specimens from normal-appearing colon were obtained from patients with colonic polyps; these served as normal control tissue. All experiments were approved by the Kurume University Medical Ethics Committee. Informed consent was given by every participant.

ELISA for phospho-JNK. To determine the amounts of phospho-JNK in tissue, we used an enzyme-linked immunosorbent assay (ELISA) for human phospho-JNK (Biosource International, Camarillo, CA). Colonic tissue samples were homogenized in phosphate-buffered saline (PBS) containing a cocktail of protease inhibitors (Sigma Chemical, St. Louis, MO) and centrifuged at 40,000 x g (21-23). Supernatants were subjected to the assay according to the manufacturer's instructions.

Immunohistochemistry for phospho-JNK. Immunohistochemistry was performed in 5-μm paraffin sections using an avidin-biotin peroxidase procedure (Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was quenched by 3% H_2O_2 for 10 min. The tissue sections were pretreated with diluted normal goat serum for 20 min, and then incubated with or without specific anti-phospho-JNK antibody raised in rabbits (Santa Cruz Biotechnology, Santa Cruz, CA). The sections were incubated with anti-rabbit biotinylated IgG for 30 min, washed with PBS, incubated with avidin/biotinylated horseradish peroxidase complex for 1 h, and washed with PBS. Slides were then stained with diaminobenzidine tetrahydrochloride substrate for 2 min at room temperature, rinsed in tap water for 5 min, counterstained with hematoxylin, and dipped in saturated lithium carbonate solution for bluing. Normal rabbit IgG diluted to an equivalent protein concentration was applied as a control in section where the primary antibody was omitted.

Western analysis. THP-1 human monocytic cells were homogenized in 2-4 ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0)-0.5% NP-40, 1 mM EDTA, 150 mM NaCl, 10% glycerol, 1 mM sodium vanadate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonyl fluoride with a protease inhibitor cocktail (Sigma Chemical). Extracts were cleared by pelleting cellular debris at 15,000 rpm at 4˚C for 15 min, and then diluted with lysis buffer to an approximate protein concentration of 2 mg/ml. Total cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were detected by immunoblotting as described previously using anti-phospho-JNK (Santa Cruz Biotechnology), anti-phospho-p38 (Cell Signaling Technology, Beverly, MA), and anti-phospho-ERK (Santa Cruz Biotechnology).

Effect of SP600125 on cytokine production in vitro. PBMC for *in vitro* studies were isolated from the heparinized blood from patients with IBD or from normal subjects by density centrifugation through Ficoll-Hypaque. These cells were washed twice in sterile phosphate-buffered saline, and then incubated with various concentrations of SP600125 at a density of 10^6 cells/ml in a complete medium consisting of RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan), 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM/l L-glutamine. Thirty minutes later, all cells were stimulated with LPS (10 μg/ml) and incubated for 15 h in a humidified environment at 37˚C with the atmosphere containing 5% CO_2. Colonic tissue specimens also were taken from IBD patients during colonoscopy. Three tissue specimens were placed on a metal grid within the wells of a 24-well tissue culture plate (Falcon 3947; Beckton-Dickinson, Lincoln Park, NJ) which contained culture medium supplemented with 10% FCS. Cultures were exposed to various concentrations of SP600125 for 15 h in a humidified environment at 37˚C including 5% CO_2. Supernatants and cultured tissues were collected and stored at -70˚C until ELISAs for TNF-α, IL-1ß, and IL-6 (R&D Systems, Minneapolis, MN).

Studies in a rat model of colitis

Induction of colitis. Eight-week-old female Sprague-Dawley rats purchased from SLC (Hamamatsu, Japan) were housed in standard wire-mesh cages. Rats received a pelleted laboratory animal formula and tap water *ad libitum*. Colitis was induced by drinking water supplemented with 2.5% (wt/vol) dextran sulfate sodium (DSS; mol wt, 5 kDa; Wako Pure Chemical Industries, Osaka, Japan). This model has been described in detail previously (24-26). The experimental protocol was approved by the Animal Research Committee of Kurume University.

Western analysis. After the colon was removed and opened longitudinally, the distal colon was separated to be homo-

genized and processed for Western blotting. Tyrosine-phosphorylation of JNK and p38 was detected with specific antibodies as described above.

Treatment protocol. SP600125 (30 mg/kg) or vehicle (40% polyethylene glycol, PEG 400, in PBS) was injected subcutaneously. The treatment was administered 2 h before the first DSS exposure and repeated every 12 h for a total of 15 doses until rats were sacrificed on day 7.

Assessment of colitis. After induction of colitis, rats were sacrificed and colons were removed. Severity of colitis was evaluated clinically, histologically, and by measuring the colon length. A clinical score was assigned based on a 0-4 rating of body weight, stool consistency, and intestinal bleeding (27). Each variable was given equal weight, with the overall clinical activity score ranging from 0 to 12. Clinical scores were measured by an investigator blinded to the animals' treatment groups. A histologic score was assigned by two pathologists blinded to treatment group who examined specimens in random order. The histologic score for each segment (cecum, proximal colon, middle colon, and distal colon) ranged from 0 to 9, and represented the sum of scores for severity of inflammation, damage/necrosis, and regeneration (28). Inflammation grades were: 0, none; 1, mild; 2, moderate; and 3, severe. Damage/necrosis was graded as: 0, none; 1, mild (superficial); 2, moderate (involving the muscularis mucose); or 3, severe (transmural, involving the muscularis propria). Regeneration grades were: 3, none; 2, focal migration and mitotic features; 1, broad, multifocal re-epithelialization; or 0, complete re-epithelialization. Finally, extent grades were: 0, none; 1, focal; 2, limited to one segment; or 3, involving more than one segment. A total histologic score, ranging from 0 to 12, consisted of the score in the distal colon plus the score for disease extent. Macroscopically, the colonic length from the cecocolonic junction to the anal verge was measured as an established inflammatory parameter in DSS-induced colitis (24-26).

Statistical analysis. Results are expressed as means ± SEM. Data were analyzed using analysis of variance (ANOVA), the Kruskal-Wallis test, the Mann-Whitney U test, and the unpaired Student's t-test as appropriate. A p-value <0.05 was considered indicative of statistical significance.

Results

JNK activation in IBD. In initial ELISA studies we determined the amounts of phospho-JNK in the colonic tissue of patients with IBD. Detectable phospho-JNK was present in tissue from every subject, and was significantly more abundant in inflamed tissue from patients with ulcerative colitis and Crohn's disease than in normal tissue from control subjects (Fig. 1A). Immunohistochemical studies were also performed to characterize patterns of phospho-JNK expression and intracellular localization in colonic mucosa. In both ulcerative colitis and Crohn's disease, we detected significant phospho-JNK immunoreactivity almost exclusively in mononuclear leukocytes in the lamina propria and epithelial cells at sites of mucosal involvement (Fig. 1B). Within cells, phospho-JNK

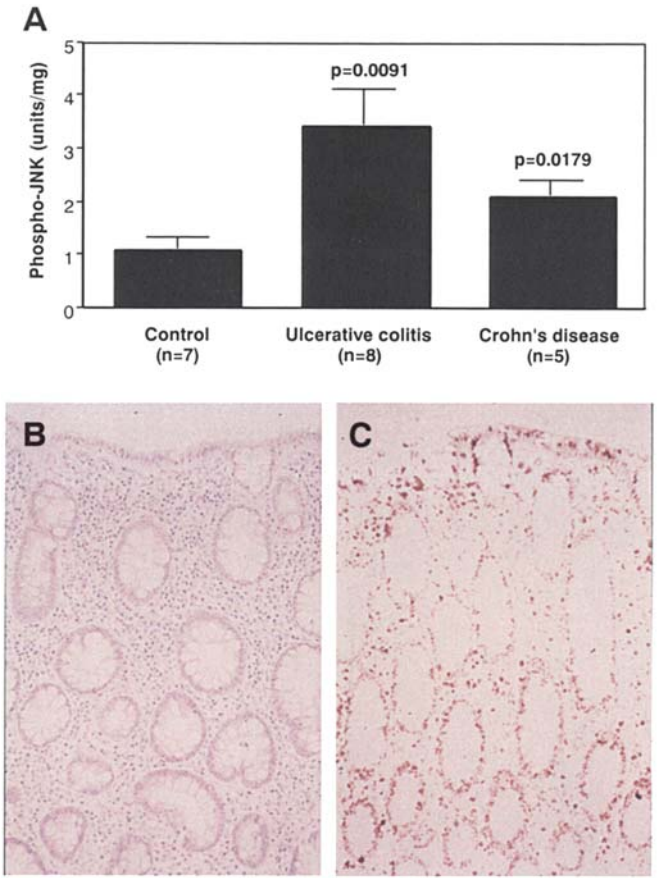

Figure 1. Phospho-JNK in patients with ulcerative colitis or Crohn's disease and normal controls. (A) Cell extracts from normal-appearing mucosa of control subjects and those from inflamed mucosa from patients with ulcerative colitis or Crohn's disease were subjected to ELISA for phosphorylated JNK. Significantly more phospho-JNK was detected in ulcerative colitis and Crohn's disease lesions than in control mucosa. (B and C) Representative immunohistochemical staining for phospho-JNK in the colonic mucosa of a control subject (B) and a patient with ulcerative colitis (C). The latter specimen showed extensive phospho-JNK immunopositivity at the nucleus of epithelial and lamina propria mononuclear cells; similar results were obtained in Crohn's disease. Normal mucosa showed little phospho-JNK expression. Control sections incubated with control IgG as opposed to specific primary antibody showed no immunoreactivity. The degree of staining was greatly reduced after preincubating the primary antibody with each recombinant protein. We analyzed more than four subjects in each group, obtaining similar results. Original magnification, x200.

was localized consistently to the nucleus. In contrast to the mucosal sites involved in IBD, we found only scattered cells immunoreactive for phospho-JNK in normal colonic mucosa (Fig. 1B) as well as in uninvolved mucosa in IBD cases (data not shown). These observations indicate that the JNK signaling pathway is activated in sites of IBD involvement.

LPS activates MAPK in cell lines. Because MAPKs regulate cytokine production in response to stress, we measured MAPK phosphorylation in the THP-1 human monocytic cell line upon exposure to LPS. As shown in Fig. 2A, LPS induced rapid phosphorylation of JNK, p38 and, to a lesser degree, ERK. We also examined whether inflammatory cytokine production in response to LPS was regulated by JNK phosphorylation by treating this cell line with the JNK inhibitor, SP600125, in addition to LPS (Fig. 2B). While LPS alone stimulated release of TNF-α and IL-1ß (but not IL-6), cultures also treated with

SP600125 showed dose-dependent reduction of release. This indicates that the JNK pathway is required for LPS-induced cytokine release by THP-1 cells.

Effect of SP600125 on cytokine release in vitro. We examined the effect of SP600125 on cytokine release from cultured tissue samples from patients with IBD. As shown in Fig. 3A, SP600125 pretreatment of PBMC from patients with ulcerative colitis and Crohn's disease as well as PBMC from normal subjects dose-dependently inhibited TNF-α, IL-1ß, and IL-6 release induced by LPS. A similar effect was observed when inflamed tissue from IBD patients was incubated with SP600125 (Fig. 3B). Release of TNF-α and IL-1ß from tissue cultured from patients with ulcerative colitis and Crohn's disease was greater than from tissue cultured from normal controls. The presence of SP600125 in cultures of inflamed tissue significantly reduced cytokine release. These data indicate that the JNK-signaling pathway regulates inflammatory cytokine release in patients with IBD.

Time-course study in the rat colitis model. We next evaluated the effect of SP600125 *in vivo* using an animal model of colitis. After induction of DSS colitis progressive weight loss, intestinal bleeding, and diarrhea were noted. To evaluate the activation of MAPK pathways in this model, phosphorylation of JNK and p38 were determined by Western blotting (Fig. 4). JNK was phosphorylated rapidly after DSS exposure and then gradually returned to control levels of phosphorylation; p38 was phosphorylated to a lesser degree.

Effect of JNK inhibition in the rat colitis model. To determine the effect of JNK inhibition on tissue damage, rats were treated with SP600125 or vehicle from 2 h before the first DSS exposure. Serial weighting of animals showed less severe weight loss in SP600125-treated rats than in controls (Fig. 5A). Clinical evaluation of colonic inflammation showed a significant beneficial effect from SP600125 on day 7 of DSS exposure (Fig. 5B). SP600125 also reduced the histologic severity of the colitis. Finally, a significant decrease in abnormal colonic shortening was evident in SP600125-treated animals. Fig. 5C shows representative histologic lesions of DSS-induced colitis and limitation of their intensity by SP600125. Destruction of the epithelial layer and glandular architecture, inflammatory infiltrates in the lamina propria, and edema of the submucosa in the distal colon were less severe in SP600125-treated rats. Thus, JNK inhibition showed a marked protective effect against experimental colonic injury.

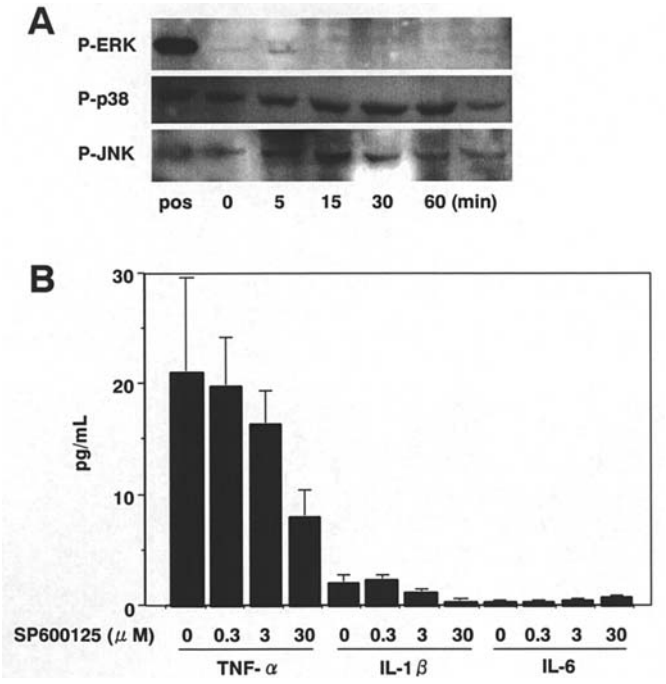

Figure 2. Studies in THP-1 monocytic cells. (A) Effect of LPS on phosphorylation of three MAPKs. Cells were stimulated with LPS (10 μg/ml) for 5, 15, 30, or 60 min. The activity of ERK, p38, and JNK was measured by immunoblotting using phosphospecific antibodies. LPS induced rapid activation of ERK, p38, and JNK. One representative experiment among four is shown. (B) Effect of SP600125 on cytokine production in THP-1 cells. Cells (10^6/ml) were preincubated with various concentrations of the JNK inhibitor, SP600125, for 30 min and then stimulated with LPS (10 μg/ml). After 15 h, culture supernatants were analyzed by ELISA for TNF-α, IL-1ß, and IL-6. SP600125 inhibited TNF-α and IL-1ß release induced by LPS. Means ± SE of triplicate samples from a representative experiment are shown.

Discussion

Based on the importance of cytokines in IBD, JNK is likely to act as a key regulator of tissue damage via its effects on AP-1 (7-14). However, previous studies of JNK's function *in vitro* and *in vivo* have been limited by lack of a specific inhibitor. A recently developed specific JNK inhibitor, SP600125 (18-20), provided us with a more precise way to investigate the role of JNK during intestinal inflammation.

We first examined whether JNK phosphorylation was greater in sites of IBD than in normal intestine. ELISA and immunostaining demonstrated that JNK was highly activated in colonic tissue with active disease. Anti-JNK immunostaining identified mononuclear leukocytes such as macrophages and lymphocytes as the main immunoreactive cell population. In these cells, the activated form of JNK was localized predominantly to the nucleus, suggesting that JNK may act during inflammation by migrating from the cytoplasm to the nucleus. These leukocytes would produce various inflammatory cytokines, lysosomal enzymes, and oxygen-free radicals in response to inflammatory stimuli in the lesion, which in turn would stimulate the recruitment of more cells. The mediators released by mononuclear cells would thus contribute to the destruction of the intestinal wall. We also found phospho-JNK in intestinal epithelial cells at lesion sites. This result is in agreement with previous reports concerning JNK activation in some colon cancer cell lines *in vitro* (29). Recently, intestinal epithelial cells have been shown to produce and release cytokines and chemokines in response to various stimuli (30-32). Cytokines secreted by epithelial cells, like those released by mononuclear cells, may influence immunocompetent cells in a manner that contributes to development of IBD.

After demonstrating the IBD-associated activation of JNK, we investigated the functional role of JNK in IBD *in vitro* by exposure of patients' PBMC and inflamed tissue to the JNK inhibitor, SP600125, as well as LPS. As expected, experiments with patient leukocytes and colonic tissues as well as the THP-1 monocytic cell line demonstrated SP600125 blockade

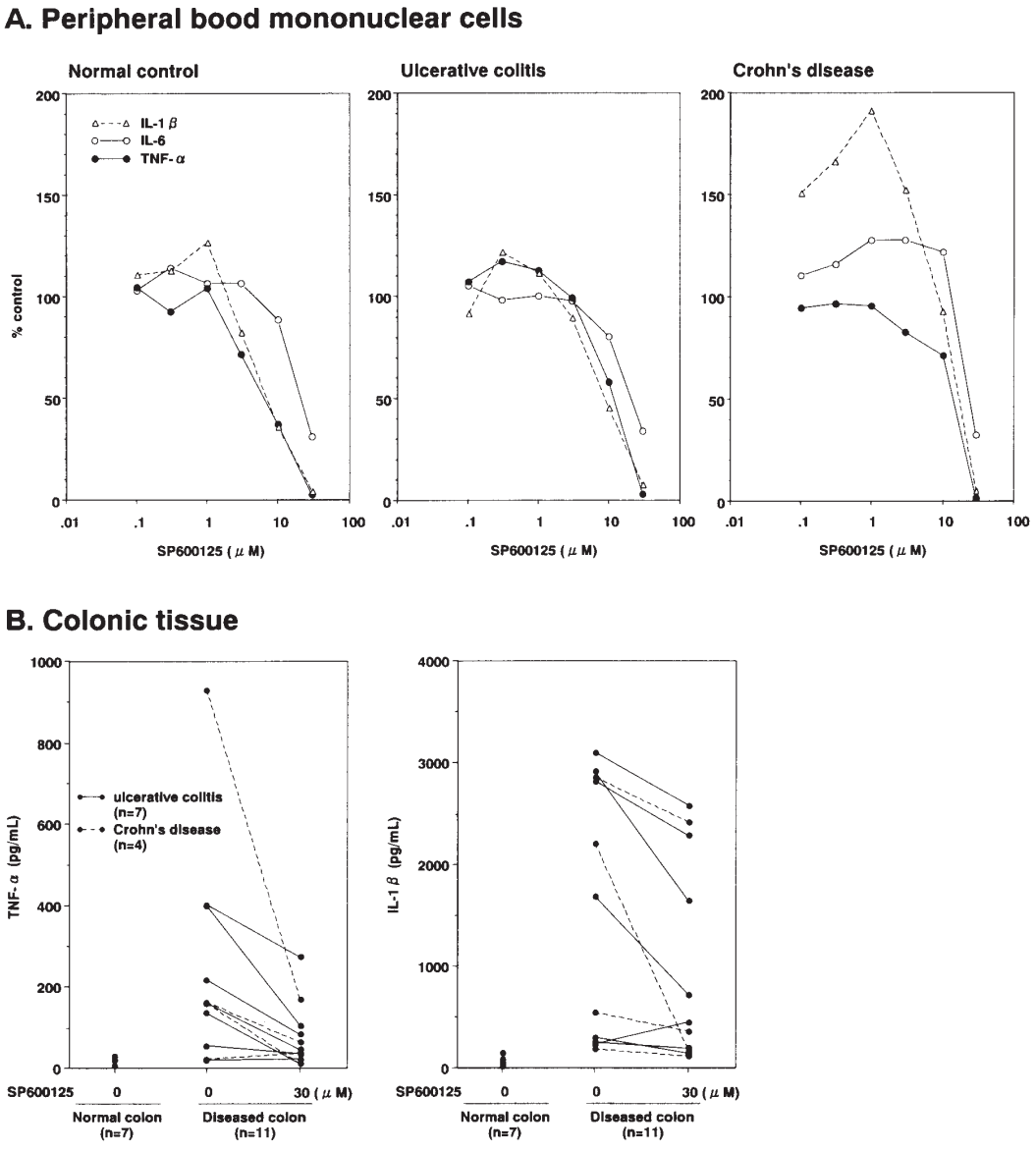

Figure 3. Effect of SP600125 on LPS-induced cytokine production. (A) Production by PBMC. Cells isolated from a normal individual, an ulcerative colitis patient, and a Crohn's disease patient were treated with the indicated doses of SP600125 for 2 h before stimulation with LPS. Supernatants were collected at 15 h after addition of LPS for ELISA to determine TNF-α, IL-1ß, and IL-6. Data are expressed as the mean of duplicate samples in a representative experiment. SP600125 blocked TNF-α, IL-1ß, and IL-6 release induced by LPS. (B) Effect of SP600125 on cytokine production by inflamed colonic tissue. Colonic tissue was obtained from IBD patients or control subjects and cultured for 15 h in the absence or presence of SP600125 (30 μM). At the end of the incubation period, culture medium was assayed for TNF-α and IL-1ß by ELISA. TNF-α and IL-1ß release was inhibited when cells were incubated with SP600125. The data represent mean cytokine concentrations in supernatants from triplicate cultures. *p<0.05, **p<0.01 vs. control (0 μM SP600125).

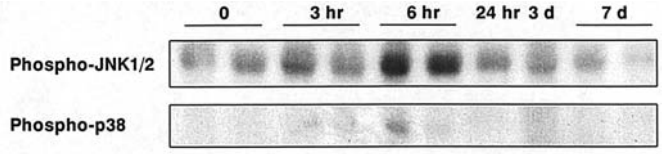

Figure 4. Time-course changes in JNK and p38 activity in colonic specimens from rats with dextran sulfate sodium (DSS)-induced colitis. Rats were sacrificed before, or 3, 6, or 24 h or 3 or 7 days after the first DSS exposure. After the colon was removed, the distal colon was separated, homogenized and processed for Western blotting.

of cytokine release. These findings indicated that the JNK signaling pathway regulates cytokine release in patients with IBD.

After demonstrating a key role of JNK in the regulation of inflammatory cytokine production *in vitro*, we evaluated SP600125's effects *in vivo*. Administration of DSS in drinking water produces inflammation limited to the colon; this model is used commonly to screen pharmacological agents for possible effectiveness against IBD. The pathogenetic mechanism of DSS-induced colitis remains undefined, although strong circumstantial evidence suggests that DSS induces colitis by damaging epithelial cells and mononuclear cells, which in turn stimulates regional inflammation through production of cytokines and other inflammatory mediators (33,34). In this study, we found that induction of DSS colitis resulted in an increase in MAPK phosphorylation, as well as in inflammatory cytokine synthesis as reported previously. The ERK signaling pathway has been associated primarily

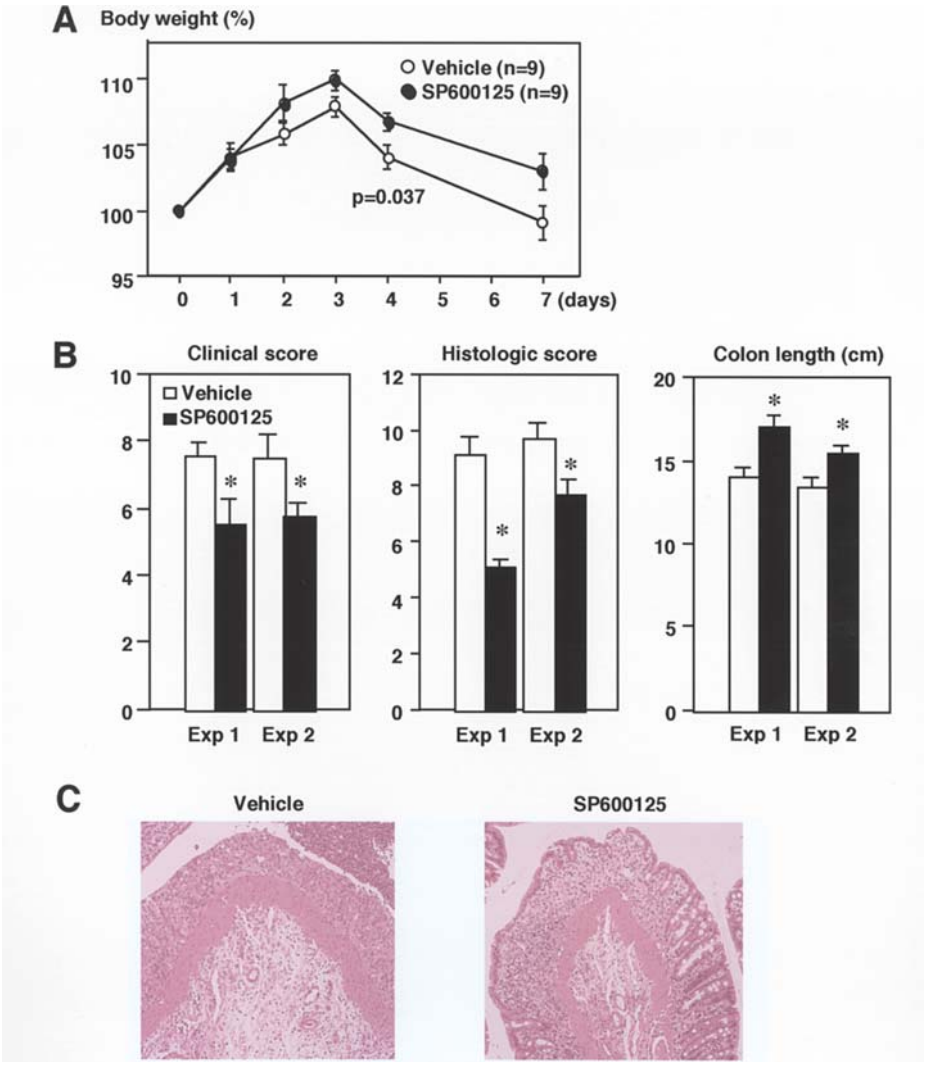

Figure 5. Inhibition of JNK prevented dextran sulfate sodium (DSS)-induced colitis in rats. Colitis was induced by drinking water supplemented with 2.5% DSS (wt/vol). (A) Changes in weight during the course of colitis. The JNK inhibitor, SP600125 (30 mg/kg), or vehicle was injected subcutaneously 2 h before the first DSS exposure and repeated every 12 h. Weight loss was less severe in SP600125-treated rats. (B) Clinical and histologic scores and colonic length in DSS-induced colitis, SP600125-treated vs. untreated rats, on day 7. Colitis was significantly less severe in SP600125-treated animals. (C) Histologic manifestations of DSS-induced colitis and their suppression by SP600125 on day 7 of DSS exposure. Destruction of the epithelial layer and the glandular architecture, inflammatory infiltrates in the lamina propria, and edema in the submucosa were less severe in the distal colon of a SP600125-treated rat (hematoxylin and eosin; original magnification, x40).

with cell growth and differentiation, while the JNK and p38 MAPK signaling pathways have been associated with inflammatory responses. Importantly, the JNK inhibitor, SP600125, markedly suppressed clinical and pathological manifestations of colitis. These findings indicate that the JNK pathway lies at a critical convergence point of cytokine regulation in colitis and suggest that SP600125 may offer therapeutic benefit. *In vivo* effects of SP600125 on inhibition of cytokine synthesis and protection against tissue injury have been evaluated in several other animal models of inflammation including adjuvant-induced arthritis and lung inflammation in rats. This is the first demonstration that SP600125 has substantial anti-inflammatory effects in a model of intestinal inflammation.

At present, the mechanism underlying the beneficial effect of SP600125 in DSS-induced colitis is not established. However, as with cultured leukocytes or colonic tissue from IBD patients, the JNK inhibitor given to rats exposed to DSS suppressed inflammatory cytokine production. Therefore, JNK blockade is likely to interfere with a cascade of events beginning with c-Jun phosphorylation and including c-jun gene expression, AP-1 binding, and cytokine gene transcription. Alternatively, JNK blockade could potentially alter immune responses since these kinases play a role in Th1/Th2 balance and T-cell activation.

Inhibition of nuclear factor (NF)-κB has also produced anti-inflammatory effects (3). Accordingly, JNK and NF-κB might complement one another in promoting inflammation. These two regulatory pathways contributing to IBD are not always linked, and different mechanisms appear to regulate their actions in intestinal inflammation. In combination with treatments to suppress other pathways such as NF-κB, inflammation might be suppressed effectively by JAK inhibition. In addition, we have reported that a negative regulator of the Janus kinases/signal transducer and activator of transcription (STAT) pathway, called suppressor of cytokine

signaling-3, can ameliorate colitis through down-regulation of STAT3 activity (25). A better understanding of how multiple transcription pathways involved in inflammatory and immune reactions are regulated may guide the development of new treatments for patients with IBD.

In summary, our study demonstrated activation of JNK in IBD as well as anti-inflammatory effects of a specific JNK inhibitor, SP600125, in this context. Activation of JNK also mediates DSS-induced colitis in rats, and SP600125 attenuated inflammation. These results identify JNK not only as a principal regulator of the inflammatory response in IBD but also as a therapeutic target.

Acknowledgements

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