

Basic Study

Restraint stress induces and exacerbates intestinal inflammation in interleukin-10 deficient mice

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Abstract

AIM: To investigate the effects of restraint stress on chronic colitis in interleukin (IL)-10 deficient (IL-10^{-/-}) mice.

METHODS: The first experiment compared the effect of restraint stress on the development of intestinal inflammation in wild-type and IL-10^{-/-} mice. Both wild-type and IL-10^{-/-} mice were physically restrained in a well-ventilated, 50 cm³ conical polypropylene tube for 2 h per day for three consecutive days. The second experiment was performed to assess the effect of restraint stress on exacerbation of colitis induced by piroxicam in IL-10^{-/-} mice. The IL-10^{-/-} mice were exposed to restraint stress for 2 h per day for 3 consecutive days, and then treated with piroxicam for 4 d at a dose of 200 ppm administered in the rodent chow.

RESULTS: In the first experiment, none of the wild-

type mice with or without restraint stress showed clinical and histopathological abnormality in the gut. However, IL-10^{-/-} mice exposed to restraint stress exhibited histologically significant intestinal inflammation as compared to those without restraint stress. In the second experiment, restraint stress significantly reduced body weight and increased the severity of intestinal inflammation assessed by histopathologic grading in IL-10^{-/-} mice. Colonic IL12p40 mRNA expression was strongly increased in mice exposed to restraint stress.

CONCLUSION: This novel animal model could be useful in future study of psychological stress in the pathogenesis of inflammatory bowel disease.

Key words: Stress; Colitis; Interleukin-10; Inflammatory bowel disease; Mouse model

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Core tip: We investigated the effect of restraint stress on the development and worsening of bowel inflammation in interleukin (IL)-10^{-/-} mice and developed a novel animal model. This is the first study to demonstrate the effect of restraint stress in inducing and exacerbating chronic colitis in IL-10^{-/-} mice. We believe that this novel animal model could be useful in future study of psychological stress in the pathogenesis of inflammatory bowel disease because this model develops chronic colitis due to interaction of genetic, immune dysregulation, microbial environment, and stress factor.

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INTRODUCTION

Stress is associated with numerous gastrointestinal disorders^[1,2]. Peptic ulcer disease is associated with stress-induced acid secretion^[3], and inpatients with psychological stress have been noted to have increased rates of irritable bowel syndrome (IBS) and functional dyspepsia^[4]. Stress is an important factor for both the development and occurrence of relapse in inflammatory bowel disease (IBD)^[5]. Patients with IBD are reported to have an increased response rate to placebo treatment, supporting the role of stress in the pathogenesis of IBD^[6,7].

It has been proposed that stress modulates intestinal inflammation in experimental models. Restraint stress was shown to induce mucosal erosion

and subepithelial hemorrhage in rats^[8]. Restraint stress exacerbates dextran sulfate sodium (DSS)-induced colitis as well as the oxidative stress damage caused by 2,4,6-trinitrobenzene sulfonic acid (TNBS)^[9,10]. Stress increases colonic permeability, facilitating bacterial translocation and activation of CD4+ T cell response^[11]. These data support the importance of stress in both the development and recurrence of bowel inflammation.

The precise pathogenesis of IBD remains obscure, but it is known to result from a complex interaction of genetic, microbial, immune dysregulation, and environmental factors^[12]. Interleukin (IL)-10 knockout (^{-/-}) mice, in which IL-10 deficiency allows for the unrestrained augmentation of a type 1 T helper (Th-1)-related immune response, represent an animal model of Crohn's disease^[13]. IL-10^{-/-} mice develops chronic colitis due to a combination of the same factors in the pathogenesis of IBD, including genetic predisposition, immune dysregulation, and microbial environment. Therefore, IL-10^{-/-}-associated colitis is more suitable than chemically-induced colitis models to evaluate the effect of stress on intestinal inflammation.

In the present study, we investigated the effect of restraint stress on the development and worsening of bowel inflammation in IL-10^{-/-} mice and developed a novel animal model.

MATERIALS AND METHODS

Mice

Seven to eight-week-old specific pathogen-free male (SPF) C57BL/6 IL-10^{-/-} mice were obtained from the Center for Animal Resource and Development (Seoul, South Korea). Age- and sex-matched wild-type (C57BL/6NCrJbGj male mice) littermates were purchased from Orient (Seongnam, South Korea). All mice were maintained on a 12-h/12-h light/dark cycle under SPF conditions. Mice had *ad libitum* access to water and standard rodent food. All animal procedures and stress protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital.

Restraint stress protocols

The first experiment compared the effect of restraint stress on the development of intestinal inflammation in wild-type (stress positive, *n* = 4; stress negative, *n* = 4) and IL-10^{-/-} mice (stress positive, *n* = 4; stress negative, *n* = 10). Both wild-type and IL-10^{-/-} mice were physically restrained in a well-ventilated, 50 cm³ conical polypropylene tube for 2 h per day for three consecutive days. The mice were euthanized 5 d after the final exposure to restraint stress.

Because the onset and severity of colitis are variable in IL-10^{-/-} mice, the second experiment was performed using piroxicam, which is known to induce rapid and uniform colitis in IL-10^{-/-} mice^[14]. The IL-10^{-/-}

mice (stress positive, $n = 4$; stress negative, $n = 6$) were exposed to restraint stress for 2 h per day for 3 consecutive days, and then treated with piroxicam for 4 days at a dose of 200 ppm administered in the rodent chow. We checked the amount of piroxicam-containing chow daily. IL-10^{-/-} mice consumed approximately 1.5-2 g of piroxicam-containing chow, which was similar in both two groups regardless of stress exposure. Mice were sacrificed on the 7th day after the first exposure to restraint stress. Mice were monitored daily for behavior, water intake, food consumption, and body weight by a researcher blinded to the restraint stress protocol.

Histopathological evaluation

Histopathological evaluation was performed as described previously^[15]. Mouse colons were immediately extracted and examined for macroscopic damage. Both the proximal and distal 2-cm portions of the colon were removed and opened longitudinally for histopathological evaluation. The resected colons from mice were fixed in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin-eosin for light microscopic evaluation or frozen in liquid nitrogen for biochemical analysis. The severity of colitis was evaluated by a pathologist blinded to the restraint stress protocol according to a previously published grading system based on mononuclear cell infiltration, epithelial cell hyperplasia, goblet cell depletion, ulceration, crypt damages, and transmural inflammation^[16]: 0 - no change from normal tissue; 1 - a few mononuclear cell infiltrates in the lamina propria with minimal epithelial hyperplasia and slight depletion of mucus from goblet cells; 2 - several multifocal, mild inflammatory cell infiltrates in the lamina propria with mild epithelial hyperplasia and mucin depletion; 3 - moderate inflammation that often involves the submucosa but is rarely transmural; 4 - intense inflammation that is sometimes transmural with marked epithelial hyperplasia, ulceration, and crypt abscesses.

Real-time reverse-transcriptase polymerase chain reaction

Quantitative real-time reverse-transcriptase polymerase chain reaction (RT-RPCR) was performed as described previously^[17]. Mouse colon tissues were homogenized and total RNA was extracted by using Trizol (GIBCO). One microgram of extracted RNA was reverse-transcribed and amplified using the SYBR green PCR master mix and an ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) with specific primers for IL-12p40 and IFN- γ . The obtained data were normalized to the level of β -actin. The following PCR primers were used: IL-12p40 forward, 5'-gga agc acg gca gaa ta-3', IL-12p40 reverse, 3'-aac ttg agg gag aag tag gaa tgg-5'; TNF- α forward, 5'-agc cca cgt cgt agc aac cac caa-3', TNF-

α reverse, 3'-aca ccc att ccc ttc aca gag-5'; β -actin forward, 5'-gtg ggc cgc tct agg cac caa-3', β -actin reverse, 3'-ctc ttt gat gat gac acg cac gat ttc-5'.

Statistical analysis

The difference between the two groups was analyzed using the Mann-Whitney *U* test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Induction of spontaneous colitis in IL-10^{-/-} mice

Before evaluating the effect of restraint stress, we assessed the basal level of intestinal inflammation in IL-10^{-/-} mice for our facility. IL-10^{-/-} mice at 6 to 7 wk of age were grossly healthy and had no intestinal inflammation. In addition, intestinal inflammation was not observed until IL-10^{-/-} mice were 11 wk old.

To assess the role of restraint stress in the inflammatory responses in the colon, 7-wk-old IL-10^{-/-} and wild-type mice were physically restrained in a well-ventilated, 50 cm³ conical polypropylene tube for 2 h per day for three consecutive days. There was no significant difference in body weight change among the four groups. None of the wild-type mice had intestinal inflammation (average colitis score 0.50 ± 0.57 and 0.75 ± 0.50 in proximal and distal colon, respectively). Mild colitis was observed in 20% of IL-10^{-/-} mice without restraint stress (average colitis score 1.20 ± 0.57 and 1.30 ± 0.50 in proximal and distal colon, respectively); the majority (80%) of IL-10^{-/-} mice without stress was completely normal. In contrast, epithelial hyperplasia, crypt abscess, ulceration, and transmural inflammation were observed in IL-10^{-/-} mice exposed to restraint stress. Histological grading showed that restraint stress significantly aggravated the overall score of colitis (average colitis score 3.00 ± 1.15 and 2.25 ± 0.96 in proximal and distal colon, respectively) (Figure 1).

Rapid exacerbations of colitis by restraint stress on piroxicam-induced colitis model in IL-10^{-/-} mice

Because the onset and severity of colitis are variable in IL-10^{-/-} mice, we performed a study using a rapid colitis model induced by piroxicam in IL-10^{-/-} mice. Body weight was significantly reduced in IL-10^{-/-} mice exposed to restraint stress 5 d after administration of piroxicam (Figure 2). The histologic examination of the proximal colons from IL-10^{-/-} mice exposed to restraint stress showed severe colitis including ulceration, transmural inflammation, and severe infiltration of inflammatory cells. In contrast, epithelial hyperplasia and mild infiltration were observed in IL-10^{-/-} mice without restraint stress. Histologic grading revealed that restraint stress exposure significantly amplified the severity score of colitis in IL-10^{-/-} mice (average colitis score 3.50 ± 0.58 and 1.60 ± 0.55) (Figure 3A). Histologic evaluation revealed less severe intestinal

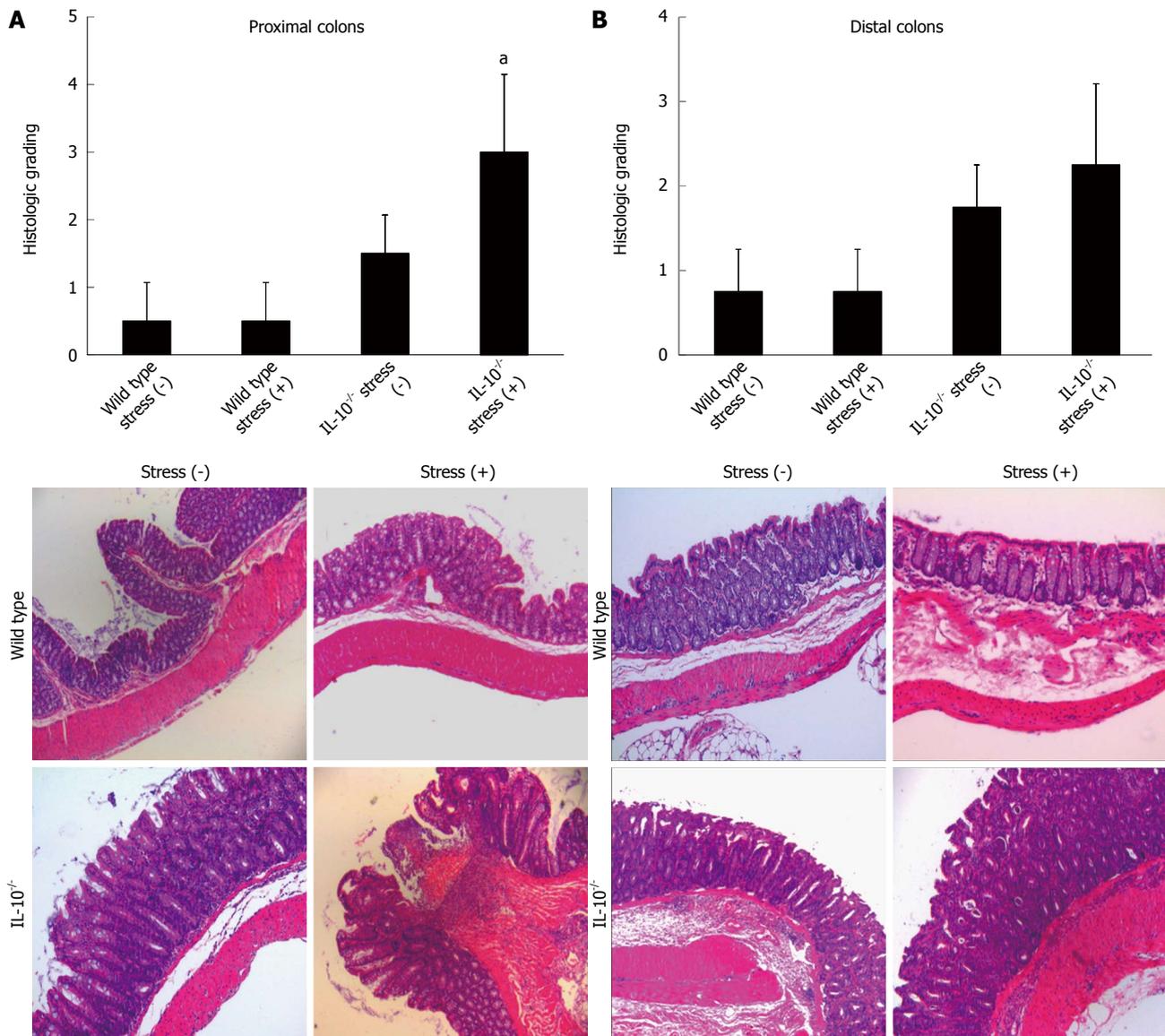


Figure 1 Histologic evaluations of (A) proximal and (B) distal colons in both wild-type and interleukin-10^{-/-} mice. Wild-type (stress positive, $n = 4$; stress negative, $n = 4$) and interleukin (IL)-10^{-/-} mice (stress positive, $n = 4$; stress negative, $n = 10$) were physically restrained in a well-ventilated, 50cc conical polypropylene tube for 2 h per day for 3 consecutive days, as described in Materials and Methods. The total histologic score was derived from the severity of total inflammation and crypt damage (mean \pm SD). Mild colitis was observed in some of IL-10^{-/-} mice without restraint stress. In contrast, epithelial hyperplasia, crypt abscess, ulceration, and transmural inflammation were observed in IL-10^{-/-} mice exposed to restraint stress. Results are representative of at least three separately examined sites (Magnification, $\times 40$). Stress (+), restraint stress for 2 h; IL-10^{-/-}: IL-10 knockout mice; ^a $P < 0.05$ vs stress negative IL-10^{-/-} mice.

inflammation in the distal colon than in the proximal colon of both groups. There was no significant difference in the histologic grade between the two groups (Figure 3B).

Restraint stress exacerbates pro-inflammatory cytokine production in IL-10^{-/-} mice

To determine whether restraint stress affects pro-inflammatory cytokine production, we performed real-time RT-PCR using extracted colons from IL-10^{-/-} mice. As shown in Figure 4, the level of IL12p40 mRNA expression was increased in the colon of the mice exposed to restraint stress. However, there was no significant difference in IFN- γ level between the two groups.

DISCUSSION

It has been proposed that IBD is caused by the interplay of host genetic factors (including barrier function and immune dysregulation), intestinal microbiota, and various environmental factors such as psychological stress^[18]. However, the precise role of psychological stress in the pathogenesis of IBD remains unclear. In an animal study, antidepressants reduce the susceptibility to the development of colitis in a model of depression induced by maternal separation^[19]. In addition, recent data demonstrated that early life stress exacerbates colitis in IL-10^{-/-} mice by inducing colonic barrier dysfunction^[20], suggesting that psychological factors such as chronic

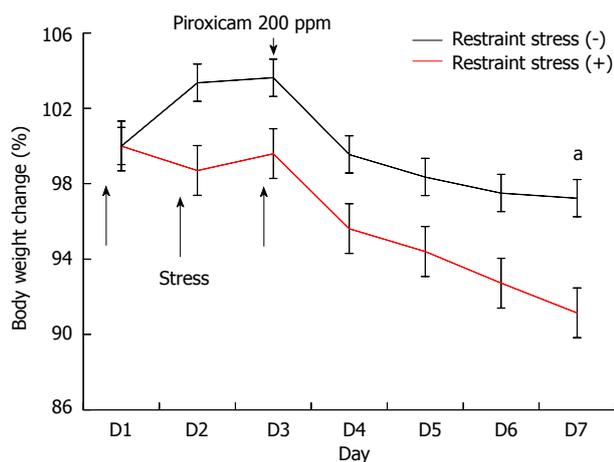


Figure 2 Body weight change in colitis model induced by piroxicam treatment. Interleukin (IL)-10^{-/-} mice (stress positive, *n* = 4; stress negative, *n* = 6) were exposed to restraint stress for 2 h per day for 3 consecutive days. IL-10^{-/-} mice were then treated with piroxicam for 4 d at a dose of 200 ppm in the rodent chow. Body weight was evaluated daily and mice were sacrificed on the 7th day after the first exposure to restraint stress. Body weight was significantly reduced in IL-10^{-/-} mice exposed to restraint stress 5 d after administration of piroxicam. **P* < 0.05 vs stress negative IL-10^{-/-} mice.

stress, adverse life events, and depression are some of the critical factors affecting the disease activity of IBD. Our data prove that restraint stress triggers the development of severe colitis in IL-10^{-/-} mice. In addition, colitis in mice exposed to restraint stress was rapidly induced by the administration of piroxicam, compared to mice without stress. Colitis induced by restraint stress in IL-10^{-/-} mice was associated with elevated pro-inflammatory cytokine. Our data therefore provide additional information regarding the role of stress in the pathogenesis of chronic colitis, suggesting that the stress-induced IL-10^{-/-} colitis model may be useful for elucidating the role of stress in IBD.

A previous study showed that restraint stress is associated with worsening of TNBS-induced colitis in rats^[9]. In addition, restraint stress increases disease activity of colitis in mice with DSS-induced colitis^[21]. These self-limiting and chemically induced colitis models are inadequate for use in studying chronic colitis, and the extent of mucosal damage may be influenced by duration or concentration of exposure of the chemical toxin. To overcome this weakness, we used IL-10^{-/-} mice, which exhibit spontaneous chronic colitis. Moreover, the histopathology of colitis in these mice is similar to that seen Crohn's disease. Our data demonstrate that restraint stress exacerbates colitis by both a clinical and histologic index in a Crohn's disease-like murine model, strengthening the evidence for the role of psychological stress in the pathogenesis of IBD.

The effect of psychological stress in intestinal inflammation is mediated by the alteration of epithelial barrier function, activation of mucosal mast cells, and hormonal changes in the hypothalamus-pituitary-adrenal gland axis^[22,23]. However, the molecular

mechanism by which restraint stress results in worsening colitis remains obscure. Our data showed no difference in the expression of IFN- γ in the colonic mucosa regardless of restraint stress. A previous study suggested that IFN- γ may not be a critical factor for stress mediated colitis, which is consistent with our data^[21]. However, severe colitis caused by restraint stress was associated with increased IL12p40 expression in the present study. IL-12 production is critical for inducing Th-1 differentiation^[24]. In addition, anti-IL-12 monoclonal antibody attenuates intestinal inflammation in IL-10^{-/-} mice^[25]. Furthermore, a human study demonstrated that psychological stress induces pro-inflammatory cytokine production and Th1-like response in stress-induced anxiety^[26]. Therefore, we believe that restraint stress may contribute to activation of Th1-related immune mechanism, resulting in exacerbations of chronic colitis. Further studies are needed to explain the precise mechanism of psychological stress in the development of colitis.

Although we have demonstrated that restraint stress aggravates intestinal inflammation, the molecular mechanism remains obscure. It is suggested that oxidative stress is one of the etiological factors of IBD^[27]. In several studies, patients with IBD demonstrated excessive reactive oxygen molecules in various specimens including colon tissues^[28-30]. More importantly, an experimental colitis study in rats showed that immobilization stress increased susceptibility to oxidative damage^[31]. Based on these results, oxidative stress seems to be an important factor in the molecular pathogenesis of stress-induced colitis. Further studies are needed to elucidate the molecular pathogenesis between stress and intestinal inflammation.

IBS and IBD are distinct diseases; however, they have some important overlapping features such as genetic factor, impaired gut barrier function, and immune activation^[32]. Interestingly, stress can activate both IBS and IBD symptoms. However, it remains unclear whether stress overlaps in the pathogenesis of both diseases. Previously, Mozaffari *et al.*^[33] used restraint stress to create an animal model of IBS. In this study, five-day restraint stress induced rapid small bowel and colonic transit. Our data showed that restraint stress aggravates the severity of histopathology in IL-10^{-/-} mice. Therefore, we believe that restraint stress animal models may be useful in investigating the common pathogenesis of IBS and IBD.

This study has several limitations. First, there are ethical considerations limiting the extent to which mice can be exposed to restraint stress, however, the Institutional Animal Care and Use Committee in our facility approved our study protocol methods after careful review. In addition, we tried to minimize the number of animals exposed to restraint stress in our study. Second, restraint stress aggravated the severity

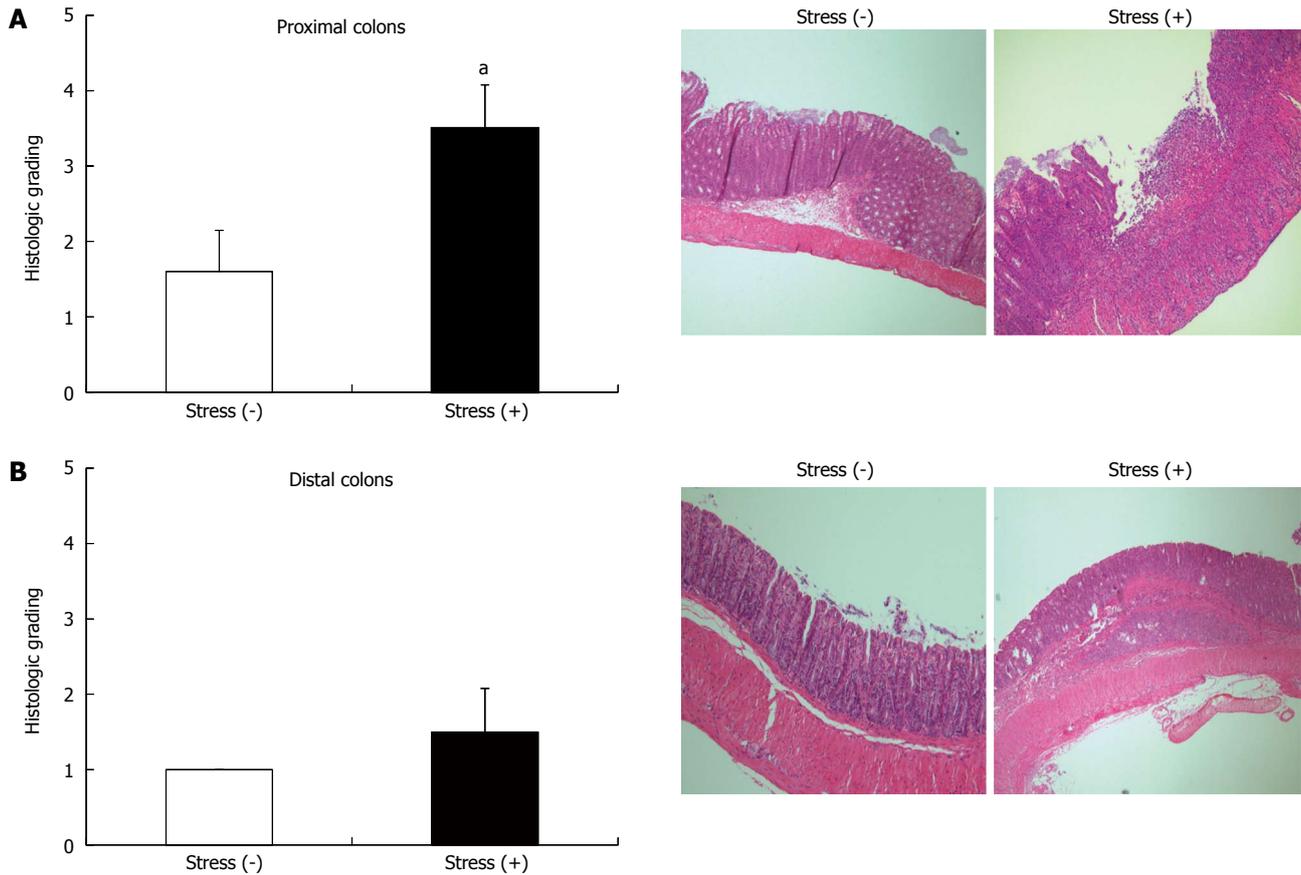


Figure 3 Histologic evaluations of (A) proximal and (B) distal colons in interleukin-10^{-/-} mouse colitis induced by piroxicam. Interleukin (IL)-10^{-/-} mice were exposed to restraint stress for 2 h per day for 3 consecutive days. IL-10^{-/-} mice were then treated with piroxicam for 4 d at a dose of 200 ppm in the rodent chow. The total histologic score was derived from the severity of total inflammation and crypt damage (mean ± SD). Mice with restraint stress exhibited severe colitis including inflammatory cell infiltration, crypt abscess, transmural inflammation, and ulceration, whereas mice without restraint stress showed epithelial hyperplasia and inflammatory cell infiltration. Results are representative of at least three separately examined sites (Magnification, x 40). Stress (+), restraint stress for 2 h. ^aP < 0.05 vs stress negative IL-10^{-/-} mice.

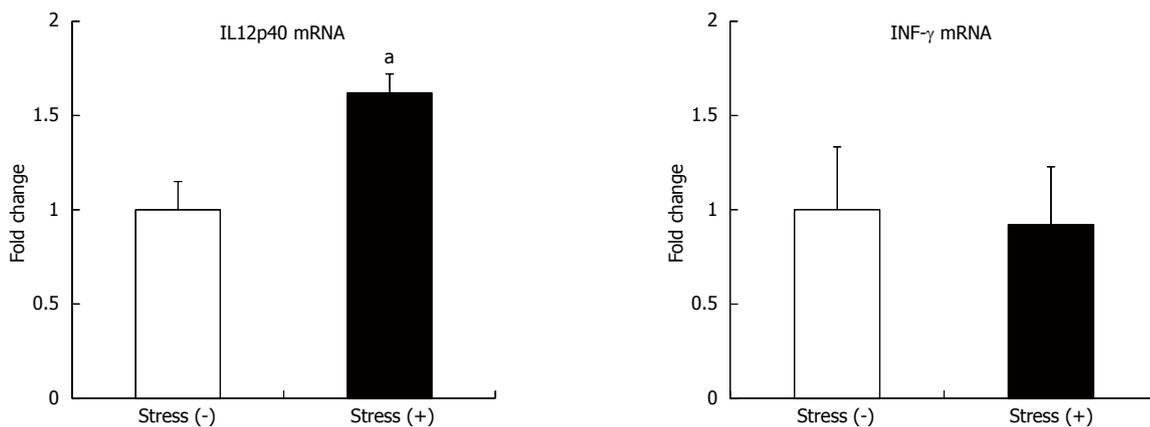


Figure 4 Restraint stress induced pro-inflammatory cytokine production in interleukin-10^{-/-} mice with colitis. Cytokine mRNA was extracted from mouse colon. Relative mRNA expression of interleukin (IL)-12p40 and IFN-γ against β-actin is expressed as the mean ± SE. The data are representative of three independent experiments. Stress (+), restraint stress for 2 h; ^aP < 0.05 vs stress negative IL-10^{-/-} mice.

of colitis in the proximal colon. As known, IL-10^{-/-} mice exhibit minor histological change in the distal colon, compared to that in the proximal colon. Although we could not confirm statistical significance, the trend toward aggravating the severity of inflammation was

shown for the distal colon. Finally, we could not provide a precise molecular mechanism by which restraint stress results in chronic intestinal inflammation.

In conclusion, our study showed that restraint stress induces and exacerbates intestinal inflammation

and pro-inflammatory cytokine production in IL-10^{-/-} mice. This novel animal model may prove useful in future study of psychological stress in the pathogenesis of IBD.

COMMENTS

Background

The precise pathogenesis of inflammatory bowel disease (IBD) remains obscure, but it is known to result from a complex interaction of genetic, microbial, immune dysregulation, and environmental factors. It has been proposed that stress modulates intestinal inflammation. The authors investigated the effect of restraint stress on the development and worsening of bowel inflammation in interleukin (IL)-10^{-/-} mice and developed a novel animal model.

Research frontiers

The authors investigated the effect of restraint stress on the development and worsening of bowel inflammation in IL-10^{-/-} mice and developed a novel animal model.

Innovations and breakthroughs

This study demonstrates for the first time that restraint stress induces and exacerbates intestinal inflammation and pro-inflammatory cytokine production in IL-10^{-/-} mice.

Applications

This novel animal model may prove useful in future study of psychological stress in the pathogenesis of IBD.

Terminology

IL-10 is an anti-inflammatory cytokine. IL-10^{-/-} mice exhibit spontaneous Crohn's disease-like colitis. IL12p40 is a cytokine for the differentiation of T-cells.

Peer-review

The authors examined the effect of restraint stress in a chronic colitis model. IL-10^{-/-} mice exposed to restraint stress exhibited histologically significant intestinal inflammation. Colonic IL12p40 mRNA expression was strongly increased in mice exposed to restraint stress. These results establish a novel model of stress-aggravated IBD-like colitis.

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