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Nafamostat mesilate attenuates colonic inflammation and mast cell infiltration in the experimental colitis

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ABSTRACT

Serine proteases are important in the pathogenesis of intestinal inflammation. Recent studies have shown that nafamostat mesilate (NM) can inhibit the colonic mucosal inflammation induced by TNBS in rats. The aim of this study was to investigate the anti-inflammatory effects of NM on a DSS-induced colitis. Colitis was induced in female BALB/c mice by 5% dextran sulfate sodium (DSS) for 6 days. NM (2 or 20 mg/kg body weight) was orally administered once a day for 6 days during treatment of the mice with DSS. The inflammatory response of the colon was assessed 1 week after DSS treatment. NM at a high dose, but not at a low dose significantly decreased disease activity index (DAI) and myeloperoxidase (MPO) induced by DSS. Furthermore, NM (20 mg/kg) inhibited the production of tumor necrosis factor (TNF)-α, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in the colonic tissues treated with DSS. The increase in chymase activity by DSS treatment was also attenuated by the administration of NM (20 mg/kg). NM (20 mg/kg) significantly decreased the colonic mucosal injury and the infiltrated mast cell number induced by DSS. These results indicate that NM might inhibit the colonic inflammation through inhibition of both chymase activity and mast cell infiltration in colon tissues of DSS-induced colitis.

1. Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory disorders of the gastrointestinal tract and include ulcerative colitis (UC) and Crohn's disease (CD) [1–3]. UC is a typical inflammatory intestinal disease characterized by erosion, mucosal ulceration, and infiltration of inflammatory cells and shows clinical manifestations that include loss of weight, diarrhea accompanied with blood and mucus, fever, gastric dysmotility, and shortening of the colon [4]. Tissues obtained from UC patients show ulceration of the mucosa, blunting and loss of crypts, as well as infiltration of inflammatory cells [5]. Moreover, prolonged and chronic UC may progress to colorectal cancer [6]. UC is believed to result from the interaction of various immune and environmental factors, but no satisfactory therapeutic program targeting UC has yet been established.

Recent studies have demonstrated that mast cells are involved in the pathogenesis of colitis [7]. The number of mast cells in the intestinal mucosa was shown to be markedly increased in patients with UC [8]. Mast cell degranulation was involved not only in the immediate hypersensitivity but also in the elicitation of delayed-type hypersensitivity including UC [9,10]. The secretory serine proteases of mast cells have been reported to play an important role in inflammatory reactions. These proteases are highly and selectively expressed in mast cells and stored almost exclusively in the secretory granules of mast cells in the form of a fully enzymatically active state. The neutral proteases released by mast cells include tryptase and chymase. Mast cell tryptase and chymase are reliable markers of mast cell degranulation. Although human colonic mucosa expresses both trypase and chymase, mouse colonic mucosa expresses only chymase [11,12]. The secretion of tryptase was significantly increased in colonic tissues of UC patients, while little is known of the actions of chymase in IBD [11]. Tryptase exhibits trypsin-like activity and the intracolonic administration of tryptase showed an induction of colonic inflammation in mice [13]. Our previous study observed that tryptase-positive mast cells expressed pro-inflammatory cytokine TNF-α in tissues obtained from UC patients [14]. Furthermore, recent studies have demonstrated that chymase plays an important role in the development of colitis via matrix metalloproteinase (MMP)-9 activation. A chymase inhibitor NK3201 significantly attenuated not only chymase activity but also colonic inflammation in DSS-treated mice [15].

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Nafamostat mesilate (NM, 6-amidino-2-naphthy-p-guanidino-benzoate dimethane sulfonate) was originally developed as an inhibitor of complements [16] and it has also been shown to inhibit the tryptase [17–19]. Based on these inhibitory profiles, this drug has been widely used for the treatment of acute pancreatitis [20]. Recent studies have shown that NM can inhibit the colonic mucosal inflammation induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in rats [21].

In the present study, the inhibitory effects of NM on colonic chymase activity, mast cell infiltration and lesions in DSS-induced colitis were examined.

2. Materials and methods

2.1. Animals and reagents

Female BALB/c mice (8 weeks old) were obtained from the SamTaco animal facility (Gyeonggi, Korea). The animal experiment was performed under the rule of Institutional Review Board (IRB). Mice were housed in a specific pathogen-free environment for at least 1 week in order to adapt to the environmental changes. DSS (mol wt; 36,000–50,000 kDa) was purchased from ICN Biomedicals (Aurora, OH). NM was purchased from BioMol (Plymouth Meeting, PA) and diluted with autoclaved saline. 5-Aminosalicylic acid (5-ASA, Sigma Co., St. Louis, MO) was used as a comparative control. The specific antibodies against iNOS and COX-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). All chemical reagents were from Sigma Co. (St. Louis, MO).

2.2. Induction of colitis by DSS

DSS was dissolved in autoclaved drinking water. Colitis of mice was induced by providing drinking water ad libitum containing 5% DSS (w/v) for 6 days as described by Okayasu et al. [22] In this model, mice were checked daily for loss of body weight, stool consistency and the presence of gross bleeding. Mice were randomly divided into groups receiving NM (2 mg/kg or 20 mg/kg), or saline (50 μl) as a negative control. NM or saline were administrated once a day for 6 days on DSS treatment. Mice were finally sacrificed and assessed after DSS treatment for 6 days. 5-ASA (150 mg/kg) was used as a comparative control.

2.3. Disease activity index (DAI)

The activity of intestinal disease was assessed through manifestations that comprised a loss of weight, diarrhea accompanied with blood and mucus, and shortening of the colon. As described by Murthy et al. [23], the DAI was obtained from a score of three major clinical signs, namely weight loss, diarrhea, and rectal bleeding, after DSS administration. (A) Body weight: Loss of body weight was calculated as the difference to body weight before experiments. (B) Diarrhea: Diarrhea was observed as mucus/fecal material adherent to anal fur. The presence or absence of diarrhea was confirmed by examination of the colon following completion of the experiment. Mice were sacrificed and the colon excised from the animal. Diarrhea was defined by the absence of fecal pellet formation in the colon and the presence of continuous fluid fecal material in the colon. (C) Rectal bleeding: The appearance of rectal bleeding was categorized as diarrhea containing visible blood and gross rectal bleeding and scored as described for diarrhea. The three major clinical signs (weight loss, diarrhea, and occult/gross bleeding) were scored separately as shown in Table 1. DAI was calculated from score of the clinical signs using the following formula: DAI = (weight loss score) + (diarrhea score) + (rectal bleeding score). The clinical parameters used here are comprehensive functional measures that are analogous to the subjective clinical symptoms observed in human UC. This method of scoring has been validated by repeated experiments. It has shown good correlation to histological healing measured as crypt scores. A significant decrease in the DAI can be considered as the end-point of successful therapy.

Table 1
Scoring of clinical signs for the disease activity index.

Score	Loss of weight (%)	Diarrhea	Occult/gross bleeding
0	None	None	Normal
1	1–5		
2	5–10	Mild	Occult
3	11–15		
4	>15	Severe, watery	Gross bleeding

Disease activity index is the mean of individually combined scores of weight-loss, diarrhea and bleeding.

2.4. Myeloperoxidase activity assay

As described by Krawisz et al. [24], the samples of colon tissue were homogenized in 20 mM phosphate buffer (pH 7.4) for 30 s and centrifuged at 15,000 *g* for 20 min. The pellet was re-homogenized in 10 volumes of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB, Sigma, St. Louis, Mo.) and 10 mM EDTA on an ice bath. The homogenate was then freeze-thawed once, sonicated for 1 min. and centrifuged at 15,000 *g* for 20 min. For purposes of assaying the myeloperoxidase activity, 50 μl of the supernatant was added to wells of a 96-well plate and mixed with 50 μl of 50 mM phosphate buffer (pH 6.0) containing 0.5% HTAB, 50 μl of o-dianisidine (0.68 mg/ml in distilled water), and 0.3 mM hydrogen peroxide to initiate the reaction. The reaction mixture was incubated for 3 min at 37 °C and the change in absorbance was measured spectrophotometrically at 450 nm. Pure human MPO (Sigma) was used as a standard. The percentage of inhibition in myeloperoxidase activity was calculated using the following equation:

$$\text{Inhibition}(\%) = \frac{(A-B)}{A} \times 100$$

where *A* and *B* represented the MPO activity in DSS-induced colitis without and with the treatment, respectively.

2.5. Enzyme-linked immunosorbent assay (ELISA)

TNF-α levels in the supernatant were measured according to the commercial instructions using the TNF-α ELISA kit (Pharmingen assay, SanDiego, CA). The ELISA plates (Falcon, Becton Dickinson Labware, NJ) were coated overnight at 4 °C with anti-TNF-α monoclonal antibody diluted in coating buffer (0.1 M sodium carbonate, pH 9.5). Coated plates were washed with PBS containing 0.05% Tween-20 and non-specific protein binding sites were blocked with assay diluent (PBS with 10% FBS) for 1 h. Each supernatant sample diluted in assay diluent was added to the wells and incubated for 2 h. One-hundred microliters of working detector (biotinylated anti- TNF-α monoclonal antibody and avidin-HRP reagent) was applied to the sample wells for 1 h. The substrate solution (tetramethylbenzidine (TMB) + hydrogen peroxidase, 100 μl) was added to each well and incubated for 30 min at room temperature in the dark. After addition of the stop solution (2 N-H_2SO_4, 50 μl), the optical density was measured at 450 nm using a microplate reader.

2.6. Western blot analysis

The distal colon tissue samples (100 mg) were homogenized in 600 μl of lysis buffer (iNtRON Biotech, Korea), incubated for 30 min on ice, and centrifuged at 15,000 *g* for 5 min. The supernatants were transferred into a fresh tube and their protein concentrations were