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Neutrophil recruitment is critical for 5-fluorouracil-induced diarrhea and the decrease in aquaporins in the colon

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ABSTRACT

Diarrhea is a common side effect experienced by cancer patients undergoing clinical chemotherapy, such as with 5-fluorouracil (5-FU). However, the precise mechanisms underlying 5-FU-induced diarrhea remain unclear. In the present study, we examined the role of neutrophil in 5-FU-induced diarrhea. Mice were given 5-FU (50 mg/kg, i.p.) daily for 4 days. Sivelestat sodium (100 or 300 mg/kg, i.p., neutorophil elastase inhibitor) or SB225002 (3 or 9 mg/kg, i.p., CXCR2 antagonist) was administered before the administration of 5-FU. Gene expression levels of aquaporin (AQP) 4 and 8, CXCL1, CXCL2, CXCL3, neutrophil elastase (Elane) and myeloperoxidase (MPO) in the colon were examined by real-time RT-PCR. The neutrophil (Ly-6G positive cell) number in the mucosa of colon was measured by flow-cytometric analysis. Administration of 5-FU induced diarrhea and decreased the expression levels of AQP 4 and 8 in the colon. Under the present conditions, the expression levels of CXCL1, CXCL2, CXCL3, the neutrophil markers Elane and MPO, as well as Ly-6G-positive neutrophils, in the colon were significantly increased by 5-FU. Neutrophil recruitment with decreased levels of AQP 4 and 8 were dramatically inhibited by either sivelestat sodium or SB225002. Furthermore, these reagents reduced the 5-FU-induced body weight loss and diarrhea. These findings provide evidence that neutrophil recruitment and neutrophil elastase may decrease the levels of AQP 4 and 8 in the colon of mice treated with 5-FU and contribute to the pathophysiology of 5-FU-induced body weight loss and diarrhea.

Introduction

Diarrhea is a common side effect experienced by cancer patients undergoing clinical chemotherapy [1,2]. 5-fluorouracil (5-FU) is widely used to treat malignant tumors due to its ability to improve the tumor-free status and survival rates [3]. However, serious side effects which include severe diarrhea [1,4,5] induced by 5-FU often necessitate a decrease in the drug dose or even a discontinuation of treatment, which may threaten the success of cancer chemotherapy.

The gastrointestinal (GI) mucositis induced by 5-FU chemotherapy is a consequence of abnormal inflammatory responses that lead to intestinal malabsorption and dysfunctions [6]. The mechanisms that underlie the chemotherapy-mediated induction of mucositis are still poorly understood, but may be associated with the production of proinflammatory cytokines such as TNF-α and IL-1β [7,8]. However, we recently reported that etanercept, a TNF-α inhibitor, significantly reduced the 5-FU-induced increase in gene expression levels of IL-1β, IL-6, IFNγ, IL-17A and IL-22 in the colon of mouse, and exacerbated 5-FU-induced diarrhea [9].

We recently reported that the genes of aquaporins (AQPs) 4 and 8 were mainly expressed in the murine colon [9]. It is widely thought that AQPs are involved in diseases that are characterized by alterations in water transport. The regulation of transepithelial fluid transport in the GI tract is based on ion transport and

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water transport by AQPs [10]. Diarrhea is a common symptom of patients with inflammatory bowel disease (IBD), and a reduction in the expression of AQPs appears to be associated with increased disease activity in patients with ulcerative and Crohn's colitis [11]. Defects in water absorption in the GI tract are important factors in the pathogenesis of diarrhea. The changes in AQP expression in diseases of the digestive system have been useful for understanding the functions of AQPs. Recently, we demonstrated that the expression levels of AQP 4 and 8 in the intestines were significantly decreased by treatment with 5-FU [9].

Growth-related oncogenes (GROs; GROα; CXCL1, GROβ; CXCL2 and GROγ; CXCL3) are all CXC chemokines, which have neutrophil-activating and neutrophil-chemoattracting properties similar to those of interleukin-8 (IL-8; CXCL8). Neutrophils are innate inflammatory cells in chronic inflammatory diseases and are attracted to the site of inflammation via chemoattractants, such as IL-8 in humans and CXCL1 or CXCL2 in mice [12]. CXCL1, CXCL2 and CXCL3 bind to the chemokine receptor CXCR2, which is predominantly expressed in neutrophils. Activated neutrophils release proteases, which contribute to tissue injury.

Neutrophil elastase is a major secretory product from activated neutrophils and a major contributor to tissue destruction in inflammatory diseases such as acute respiratory distress syndrome (ARDS), lung emphysema, and so on [13,14]. It has been demonstrated that the plasma neutrophil elastase level is associated with the clinical activity of ulcerative colitis. Sivelestat sodium, a specific synthetic inhibitor of neutrophil elastase has been shown to have a protective effect against neutrophil-mediated tissue injury in some animal models, including lung injury, neurologic damage after spinal cord injury, and collagen-induced arthritis [15–18]. In the present study, we speculated that neutrophilic inflammation may cause diarrhea with changes in AQP expression. To investigate the role of neutrophils in 5-FU-induced diarrhea, we examined the effects of sivelestat sodium and a selective CXCR2 antagonist on the development of diarrhea and the downregulation of AQP4 and 8 expression with the administration of 5-FU in the mouse.

Materials and methods

Animals

Male C57BL/6J mice (8–9 weeks of age, 23–27 g) were used. All experiments were approved by the Animal Care Committee at Hoshi University (Tokyo, Japan).

Treatment protocol

Mice were given a single intraperitoneal injection of 5-fluorouracil (5-FU; 50 mg/kg) daily for 4 days, with saline (vehicle) used as a control (Fig. 1A). Twenty-four hour after the final injection of 5-FU (Day 3), animals were killed under deep anesthesia with isoflurane, and the proximal colon, transverse colon, and distal colon were removed, washed with cold saline, and stored in TRI Reagent™ (Sigma–Aldrich) at −80 °C. Sivelestat sodium (100 or 300 mg/kg, Ono Pharmaceutical Co., Ltd.) or SB225002 (3 or 9 mg/kg, CXCR2 antagonist, Cayman Chemical Co., Ltd.) was administered intraperitoneally 30 min before the administration of 5-FU on Days 0–4. Previous study indicated that sivelestat sodium (100 mg/kg/day, i.p.) prevented the development of DSS-induced colitis in mice. Therefore we used doses of 100 and 300 mg/kg/day, i.p. [19]. On the other hand, Manjavachi [20] previously showed that the intraneural injection of CXCL1 in the mouse sciatic nerve elicited long-lasting mechanical hyperalgesia, which was prevented by SB225002 (3 mg/kg, i.p.). Therefore, 3 and 9 mg/kg, i.p. of SB225002 were used in this study.

Diarrhea assessment

A diarrhea score was determined for each mouse. Diarrhea assessment was performed by four blind investigators, and their data were averaged. The severity of diarrhea was scored using the following scale, 0: normal (normal stool), 1: minimal (soft stool), 2: slight (slightly wet and soft stool), 3: moderate (wet and unformed stool with moderate perianal ~~a~~ staining of the coat), 4: severe (watery stool with severe perianal staining of the coat). The incidence of each diarrhea score (0–4) and the average diarrhea score were used to evaluate the severity of diarrhea.

Real-time RT-PCR

Gene expression levels of AQP 4 and 8, CXCL1, CXCL2, CXCL3, Elane and MPO were examined by real-time RT-PCR as described previously [9]. Briefly, total RNA was extracted from various tissues with a one-step guanidinium–phenol–chloroform extraction procedure using TRI Reagent™ (Sigma–Aldrich). cDNAs were prepared from total RNA (1.0 μg) by using QuantiTect Reverse Transcriptase (Qiagen, Germany) after incubation with gDNA wipeout buffer at 42 °C for 3 min to remove contaminating genomic DNA. The reaction mixture (2 μL) was subjected to PCR (50 nM forward and reverse primers, Fast SYBR Green Mastermix; Applied Biosystems) in a final volume of 10 μL. The PCR primer sets used are shown in Table 1. The thermal cycle profile used was (1) denaturing for 30 s at 95 °C, and (2) annealing for 30 s at 60 °C. PCR amplification was performed for 40 cycles. Data are expressed as the expression relative to GAPDH mRNA as a housekeeping gene using the $2^{-\text{deltadeltaCT}}$ method.

Flow cytometry analysis

Colonic mucosal layer, containing epithelial, goblet, immune and various cells, obtained from mouse was incubated in Hanks' balanced salt solution containing 10 mM HEPES (pH7.3), 1 mg/ml collagenase D (Roche Diagnostics, Penzberg, Germany) and 5 μg/ml DNase I (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C for 20 min with gentle agitation, and filtered. The cells were incubated with a phycoerythrin-labeled anti-mouse Ly-6G antibody (Miltenyi Biotech, Auburn, CA, USA) and a fluorescein isothiocyanate-labeled anti-mouse Epcam antibody (Miltenyi Biotech, Auburn, CA, USA), and subjected to flow cytometry analyses using FACS Verse (BD, Franklin Lakes, NJ, USA).

Immunohistochemical study

Mouse colonic tissue preparation and immunohistochemical procedures were performed as described by Matsumoto et al. [21]. In the frozen section of mouse distal colon, Ly-6B.2 immunoreactivities were detected by indirect staining with rat Ly-6B.2 (1:100; AbD Serotec, Raleigh, NC, USA), respectively. To visualize the each marker labeling, sections were then incubated with fluorescein tetramethylrhodamine isothiocyanate (1:400; Jackson Immunoresearch Laboratories, West Grove, PA, USA). In control experiments, the neutrophil antibody was omitted from the staining procedures to verify the specificity of the staining. No immunolabeling was observed in these controls.

Statistical analysis

The statistical significance of differences was determined by an unpaired Student *t*-test or one-way analysis of variance (ANOVA) with the Bonferroni/Dunn post hoc-test. A value of $p < 0.05$ was considered significant.