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Effects of the endothelin converting enzyme inhibitor SM-19712 in a mouse model of dextran sodium sulfate-induced colitis

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Abstract

Ingestion by mice of dextran sodium sulfate (DSS) induces colonic vasoconstriction and inflammation, with some of the effects potentially mediated by the vasoconstrictor endothelin-1 (ET-1). In this study, mice given 5% 40 kD DSS for 5–6 days had elevated colonic immunostaining for ET-1 and platelet endothelial cell adhesion molecule-1 (PECAM-1). Increased ET-1 can induce microvascular constriction; however, the increase in PECAM-1 is consistent with angiogenesis that could decrease flow resistance. Our measurements of intestinal blood flow, via infused microspheres, suggests that these two factors may offset each other, with only a nonsignificant tendency for a DSS-induced decrease in flow. Daily administration of the endothelin converting enzyme inhibitor SM-19712 (15 mg/kg) attenuated DSS-induced increases in colonic immunostaining of ET-1 and PECAM-1. SM-19712 attenuated histologic signs of tissue injury and inflammation induced by DSS, and decreased the extent of loose stools and fecal blood. However, the inhibitor did not significantly decrease DSS-induced colon shortening or tissue levels of myeloperoxidase (an indicator of neutrophil infiltration).

Keywords

endothelin converting enzyme; vasoconstriction; colon; inflammatory bowel disease

Introduction

Endothelin-1 (ET-1) is one of several vasoconstrictors that may play a role in the progression of Crohn's Disease and ulcerative colitis, conditions categorized as inflammatory bowel disease (IBD). Elevated plasma (1) and tissue (2,3) levels of the potent vasoconstrictor have been found in IBD patients, with the elevations speculated to induce hypoxia (2,3) as a consequence of decreased blood flow. As described by Hulten et al. (4) over 30 years ago, decreases in blood flow are found more frequently in chronic and mild IBD and in the late fibrosing stage of Crohn's Disease, while increases in blood flow can be found in severe IBD.

Animal models of IBD, in which blood flow has been reported to decrease, have implicated a role for ET-1 in the resulting inflammation. In the trinitrobenzenesulfonic acid (TNBS) model in rats, several studies (5–8) have investigated the potential effectiveness of the non-

specific endothelin receptor antagonists bosentan and Ro 48-5695, with the results indicating a dose- and time-dependent ability to inhibit neutrophil infiltration (measured by the activity of myeloperoxidase, MPO) and to reduce histologic signs of tissue injury. The role of ET-1 has not been characterized to the same extent in another widely used animal model of colitis, ingestion of dextran sodium sulfate (DSS), although Anthoni et al. (9) found that bosentan attenuated DSS-induced colonic inflammation and leukocyte-endothelial cell adhesion in mice, while restoring venular blood flow.

The DSS model of colitis induces several microvascular dysfunctions, including leukocyte and platelet adhesion to the venules, arteriolar constriction, deficient endothelium-dependent arteriolar dilation, and an increase in microvascular density (9–13). While constriction increases vascular resistance of individual arterioles, angiogenesis should decrease overall microvascular resistance (due to the greater number of capillary pathways), with these two contrasting effects on resistance influencing the overall blood flow to the colon. While flow in individual arterioles decreases in response to DSS (11,12), the overall colonic blood flow rate could hypothetically stay the same or even increase due to the angiogenesis, and therefore flow is measured in the current study to address this question.

ET-1 is produced in a multi-step process in which the prepropeptide is cleaved to form Big ET-1, which is then converted to ET-1 by endothelin converting enzymes (14). The endothelin converting enzyme (ECE) inhibitor SM-19712 has been found to effectively reduce injury induced by ischemia-reperfusion of the rat kidney (15) and rabbit heart (16). However, to our knowledge, the effectiveness of this or any other ECE inhibitor has yet to be tested in animal models of colitis, and is a primary goal of this investigation. In the current study, we administer the ECE inhibitor SM-19712, and measure the following endpoints: intestinal blood flow, microvascular density, a disease activity index (stool consistency, fecal blood, body weight loss), colonic shortening, histologic signs of injury, and colonic neutrophil infiltration (MPO activity).

Materials and Methods

Animals

C57BL/6 mice weighing ~28 grams (purchased from Jackson Labs; Bar Harbor, ME) were given filter purified (Millipore Corp., Bedford, MA) drinking water ad libitum. In the four groups of mice, the drinking water contained 0 or 5% (wt/vol) dextran sodium sulfate (DSS; 40 kD; ICN Biomedicals, Aurora, OH) and SM-19712 [4-Chloro-N-[[[4-cyano-3-methyl-1-phenyl-1H-pyrazol-5-yl] amino] carbonyl] benzenesulfonamide sodium salt; Sigma, St. Louis, MO) at doses of 0 or 15 mg/kg/day. These four groups are referred to as control (N=30), control + SM-19712 (N=16), DSS (N=31), and DSS + SM-19712 (N=29). The numbers of mice in each group were divided into different measurement protocols (blood flow, immunostaining, myeloperoxidase activity, etc). The mice of the four groups were kept on the drinking water protocol for 5–6 days.

Immunohistochemistry

After the animal was anesthetized i.p. with 150 mg/kg ketamine and 10 mg/kg xylazine, the entire colon was surgically removed and put in cold PBS. The colon was prepared for histology using the 'Swiss roll' method (17). The colon was cut lengthwise and laid flat on a cotton pad soaked with cold water in a petri dish. The flattened section was flushed with cold PBS (Sigma, St. Louis, MO) to remove fecal matter and fixed with 1 ml 4% para-formaldehyde (FD NeuroTechnologies, Inc. Baltimore, MD) for 10 minutes. The colon was separated in half longitudinally using a no. 22 surgical blade. With forceps, one-half of the tissue was rolled longitudinally, with the mucosa outwards, around a wooden stick (the base

of a cotton-tip applicator). The rolled tissue was then immersed in OCT compound solution (Skura Finetechnical Cl., Ltd., Tokyo, Japan) for frozen blocks, and placed overnight in a -80°C freezer. Frozen specimens were cut in 5 μm thickness slices with a cryostat (Leica CM 3050 S, Nussloch, Germany) for immunostaining of endothelin-1 and PECAM-1. The other half of the colon was rolled and fixed in 4% para-formaldehyde overnight and imbedded in a paraffin block for H&E staining.

Frozen sections on the slides were washed in PBS plus 0.25% Triton[®] X-100 (Sigma, St. Louis, MO) 3 times for 5 minutes each, then incubated with 10% normal goat serum with 1% BSA (Bovine Serum Albumin, Sigma, St. Louis, MO) in PBS for 2 hours at room temperature to block non-specific binding. The sections were incubated overnight at 4°C with the primary antibody, rabbit anti-ET-1 polyclonal antibody (T-4050, Peninsula Laboratories, Inc., Belmont, CA), diluted 1:2000 in PBS with 1% BSA. The sections were rinsed in PBS plus 0.25% Triton[®] X-100 3 times for 5 minutes each, then incubated for 1 hour at room temperature in the dark with fluorophore-conjugated secondary antibody, goat pAb to Rabbit IgG (FITC; ab 6717-1, Abcam Inc., Cambridge, MA), which was diluted to 1:1000 in PBS with 1% BSA. After rinsing with PBS 3 times for 5 minutes each, the sections were mounted with a coverslip and Vectashield mounting medium (Vector, Burlingame, CA). For comparative analysis, a section from each animal was labeled only with the secondary antibody to serve as a negative control.

For PECAM-1 staining, the same procedures were used, with the exception of the primary and secondary antibodies and their dilutions. The primary and secondary antibodies, respectively, were rat anti-mouse CD31 (550274, BD Biosciences, Franklin Lakes, NJ; dilution 1:200) and goat pAb to rat IgG (FITC; ab7093-100, AbCam, Cambridge, MA; dilution 1:250).

Immunohistochemistry analysis

Images of each tissue section were collected with a digital camera (CoolSNAP ES; Photometrics, Tucson, AZ) attached to a fluorescence microscope (Eclipse E600FN; Nikon Instruments Inc., Melville, NY) using a 10x objective, scanning from the proximal to distal end of the colon. The captured images were saved and analyzed using NIS Elements Basic Research software (Nikon Instruments Inc., Melville, NY). The histograms of each image were exported to Microsoft[®] Excel (Microsoft Corporation, Redmond, WA), tabulating the number of pixels brighter than the average background value. The tissue had a low level fluorescence exceeding this background, and therefore, the number of pixels exceeding the average background value was defined as the area of tissue (A_T). The specific staining of the secondary antibodies was brighter than the tissue fluorescence, and the area of pixels stained with the secondary antibody for ET-1 (A_E) or PECAM-1 (A_P) was calculated as the number that exceeded the tissue fluorescence threshold of the corresponding negative control slide (no secondary antibody). This threshold was defined as the gray value that exceeded 95% of the pixels within the tissue of the negative control. The ratios of A_E/A_T and A_P/A_T are used to represent the extent of staining for each.

Microsphere determination of blood flow rate

Under anesthesia (150 mg/kg ketamine and 10 mg/kg xylazine), fluorescent microspheres (Invitrogen; Carlsbad, CA) 10 μm in diameter were injected via the right carotid artery into the left ventricle. The cannulated right femoral artery served as a reference, with blood withdrawn at $\sim 60 \mu\text{l/min}$. The microspheres were infused at a rate of $60 \mu\text{l/min}$, with 200,000 beads injected; following the microspheres the cannula was flushed with 300 μm saline. The ileum and colon were excised and weighed, and the tissue and reference blood sample fluorescence were extracted by filtration as described by Raab et al. (18). The flow

rate (ml/min/g) was calculated as $(I_i)(R)/(I_{ref})$, and divided by the tissue weight, where I_i is the fluorescence intensity of the sample, R is the reference withdrawal rate (ml/min), and I_{ref} is the fluorescence intensity in the reference blood sample.

Histology scoring

Slides stained with H&E were scored in a blinded fashion in a method similar to that described by Dieleman et al. (19), where severity of inflammation (0–3 scale), depth of injury (0–3 scale), and crypt damage (0–4 scale) are multiplied by a 1–4 integer representing the percent involvement of the colonic wall (1: 0–25%; 2: 26–50%; 3: 51–75%; 4: 76–100%), for a maximum total score of 40.

Disease Activity Index

At the end of the 5–6 day protocol, mice were evaluated using a disease activity index similar to that described by Cooper et al. (20). Three parameters (stool consistency, fecal bleeding, and weight loss) were each evaluated on a 0–4 point scale and averaged for an overall disease activity index. Stool consistency was graded 0 for no diarrhea, 2 for loose stools that do not stick to the anus, and 4 for liquid stools that do stick to the anus. The presence of fecal blood was graded 0 for none, 2 for moderate, and 4 for gross bleeding. Weight loss was assigned a value of 0 if body weight remained within 1% of baseline or higher, 1 for a 1–5% loss, 2 for a 5–10% loss, 3 for a 10–15% loss, and 4 for a loss greater than 15%. At the end of the protocol, animals were sacrificed with an overdose of pentobarbital (150 mg/kg).

Tissue myeloperoxidase activity

Tissue was frozen at -80°C for subsequent measures of myeloperoxidase (MPO) activity, using O-dianisidine (Sigma) as a substrate. Details of this method have been given previously (21). Activity was expressed as units/g, with a unit defined as the 460 nm light absorbance following a 10-minute reaction period, with the reaction terminated by the addition of sodium azide.

Statistics

Multiple groups were compared using analysis of variance (ANOVA) with Student Newman Keuls post-hoc corrections (GraphPad Instat Software, San Diego, CA). Values are presented as means $\pm$ standard error.

Ethical Considerations

All animal procedures were approved by the Institutional Animal Care and Use Committee.

Results

As shown in Figure 1A, DSS induces a significant increase in ET-1 immunostaining ($p<0.01$), which was inhibited by co-administration of SM-19712 ($p<0.05$). Immunostaining for PECAM-1 demonstrated a similar pattern, with a significant increase ($p<0.001$) partially inhibited by SM-19712 by ~40% ($p<0.05$; Fig 1B).

Ileal and colonic blood flow rate, measured via infused microspheres, did not change significantly following DSS treatment with or without SM-19712 (Fig 2). Although mean arterial blood pressure (measured via a carotid arterial cannula) tended to decrease in the DSS groups, the change was not statistically significant (Fig 3).

As shown in Figure 4, blinded histological scoring indicated major injury and inflammation induced by DSS ($p < 0.001$ vs controls), which was attenuated significantly by SM-19712 by ~40% ($p < 0.001$). SM-19712 also provided protection from the incidence of loose stools (Fig 5A) and fecal blood (Fig 5B), and tended to decrease the DSS-induced weight loss (Fig 5C). The disease activity index (comprising loose stools, fecal blood, and weight loss; Fig 5D) was significantly decreased by SM-19712 in the DSS mice. Although the data in Fig 5C suggests only a tendency for SM-19712 to protect against weight loss, this 0–4 integer scale obscures the actual 15–25% difference between the untreated and SM-19712-treated DSS mice. As shown in Figure 6, when the weights are presented as the % loss during the 5–6 day protocol, statistically significant improvements in the SM-19712 mice are found ($p < 0.01$ at day 5 and $p < 0.05$ at day 6).

The presence of tissue myeloperoxidase activity (an indicator of neutrophil infiltration) was increased substantially by DSS, with the increase not significantly affected by SM-19712 co-administration (Fig 7). SM-19712 did not protect the colon from DSS-induced shortening (Fig 8).

Discussion

Our study demonstrates benefit from an administration of an endothelin converting enzyme inhibitor in a DSS mouse model of colitis. SM-19712 was able to attenuate the extent of loose stools, fecal blood, weight loss, histologic signs of injury, and angiogenesis. With the exception of loose stools, these attenuations were partial, in general $\leq 50\%$, indicating that 15 mg/kg/day SM-19712 was not able to completely inhibit the inflammatory response. Two markers of inflammation were not attenuated significantly by the drug (MPO activity, colonic shortening).

The inability of SM-19712 to attenuate tissue levels of MPO is similar to the findings reported by Deniz et al. (5) in a rat model of TNBS administration. In their study, TNBS induced large increases in MPO and macroscopic damage to the colon: administration of endothelin receptor antagonists (30 mg/kg/day bosentan; 60 μ g/day BQ485) were able to attenuate the injury, while having no effect on tissue MPO. Therefore, eliminating tissue MPOs do not seem to be a requirement for improving the condition of the colonic tissue in these models. However, the TNBS-induced MPO levels can be attenuated with very large doses and/or pretreatment with bosentan. Hogaboam et al. (7) found decreases in MPO with 30 and 60 mg/kg daily bosentan (with the doses begun 24 hours prior to TNBS), but only with 60 mg/kg when bosentan was not pre-administered. At an even higher dose (not started prior to TNBS), Gulluoglu et al. (6) found that 100 mg/kg/day bosentan could attenuate decreases in TNBS-induced colonic MPO. A more potent receptor antagonist, Ro 48-5695, was able to attenuate TNBS-induced increases in MPO by ~50% at a dose of 10 mg/kg/day.

ET-1 may have some role in human IBD, based on reports of elevated plasma (1) and tissue (2,3) levels, as well as reports of vasoconstriction during certain phases of the disease. However, during severe bouts of the disease, blood flow can increase, rather than decrease, and the increase in ET-1 may fluctuate. In contrast to other reports, McCartney et al. (22) found that endothelin (combined ET-1 & ET-2) levels were decreased in patients with active IBD. However, this decrease was not observed when their assay was repeated with inhibition of peptidase, an enzyme that could decrease the levels of protein. Their general conclusion was against a role for endothelins in human IBD, in part based on the unchanged ET-1/2 levels in human colonic biopsies following treatments that alleviate symptoms of the disease (5-aminosalicylic acid, dexamethasone, the selective cyclooxygenase-2 inhibitor L-745377, and indomethacin). However, indomethacin tended to decrease ET-1/2 by more than 50% in ulcerative colitis patients, indicating that their statistical power may have been

insufficient to detect a fairly substantial change. It may not be warranted to dismiss the possibility of ET-1 involvement, especially in the phases of IBD in which blood flow is substantially reduced.

The TNBS model of colitis involves a dramatic decrease in colonic blood flow. Of interest in the previously mentioned study by Deniz et al. (5) was the ability of the endothelin receptor antagonists (bosentan, BQ485) to restore colonic blood flow, suggesting a possible role for ET-1 in the TNBS-induced vasoconstriction. In response to TNBS, blood flow decreased from > 0.2 ml/min/g to < 0.1 ml/min/g, but was restored to ~0.15 ml/min/g with either bosentan or BQ485.

Based on intravital microscopy experiments that show substantial DSS-induced vasoconstriction and decreases in blood flow in individual arterioles of the ileum and proximal colon (11,12), an overall decrease in intestinal blood flow in the DSS protocol could be predicted. However, in the current study, we only noted a small decrease in blood flow (measured by microspheres) that did not reach statistical significance. The lack of decrease is likely due to the increase in vascularity, as our measurements of an increase in PECAM-1 agree with the previous report by Chidlow et al. (10). Angiogenesis provides a decrease in the resistance to blood flow, which appears to partially offset the arteriolar constriction. The degree to which ET-1 contributes to DSS-induced vasoconstriction has yet to be determined.

In summary, the endothelin converting enzyme inhibitor SM-19712 was able to attenuate DSS-induced increases in loose stools, fecal blood, weight loss, histologic signs of inflammation, and immunostaining of PECAM-1. The ability of SM19712 to inhibit the DSS-induced increase in PECAM-1 is worth noting, due to the role that angiogenesis might play in the progression of colitis.

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Figure 1. Immunostaining for ET-1 and PECAM-1 in colonic tissue of control and DSS mice, and of DSS mice given daily administration of SM-19712. N=7 each for ET-1, and N=11 each for PECAM-1. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs control mice; + $p < 0.05$ vs DSS mice.

Figure 2. Blood flow rate in the ileum and colon of control and DSS mice with and without SM-19712. In both panels, from left to right N= 10, 7, 11, and 9. No statistical differences between groups were found.

Figure 3. Mean arterial pressure in control and DSS mice with and without SM-19712. From left to right N= 11, 11, 7, and 8. No statistical differences between groups were found.

Figure 4. Histological scoring in control and DSS mice and in DSS mice given daily administration of SM-19712 (N=7 each). *** $p < 0.001$ vs control mice; +++ $p < 0.001$ vs DSS mice.

Figure 5.

Indices (0–4 scoring) of (A) loose stools, (B) fecal blood, and (C) weight loss in control and DSS mice with and without SM-19712. Panel D averages the three indices in a disease activity index. In each panel, from left to right N= 30, 16, 31, and 29. ***p<0.001 vs control mice; +++p<0.001 vs DSS mice.

Figure 6.

Weight loss over the 5-day (A) and 6-day (B) protocols expressed as a percentage. In panel A, N = 19, 16, 20, and 18 from left to right; N = 11 for each group in panel B. ***p<0.001 vs control mice; +p<0.05 and ++p<0.01 vs DSS mice.

Figure 7. Myeloperoxidase activity in colonic tissue of control and DSS mice with and without SM-19712 (N=9 each). *p<0.05 vs control mice.

Figure 8. Colon weight (A), length (B), and weight-to-length ratio (C) in control and DSS mice with and without SM-19712. In each panel, from left to right N= 19,16, 20, and 18. *p<0.05, **p<0.01, and ***p<0.001 vs control mice.