Regular Article

Metabolism of Dextran Sulfate Sodium by Intestinal Bacteria in Rat Cecum Is Related to Induction of Colitis

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Ulcerative colitis induced by dextran sulfate sodium (DSS) is one of the most widely used experimental animal models. However, the mechanism responsible for the pathogenesis of the colitis is still unclear. The aim of the present study was to clarify the events occurring after administration of DSS to rats focusing on the relationship between the intestinal bacterial metabolism of DSS and the intestinal mucosal lesions in the model. Within 2 d after DSS administration, severe injury of the cecal mucosa was evident, together with bloody feces and blood in the cecum. However, these lesions were repressed by administration of antibiotics. On the other hand, DSS was found to be metabolized under anaerobic conditions upon incubation with cecal content *in vitro*, first being desulfated and then undergoing carbohydrate moiety degradation. However, no such metabolic process occurred when cecal content from rats that had been administered antibiotics was employed. These results indicate that the initial step of DSS-induced ulcerative colitis is lesioning of the cecal mucosa, which is related to metabolism of DSS by intestinal bacteria.

Key words dextran sulfate; ulcerative colitis; cecum; metabolism; rat

Inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis (UC) are multifactorial disorders whose etiologies are still unclear. Although numerous reports have addressed pathologic aspects, focusing on the immune system, genetic susceptibility, environment, intestinal microbiota, and other factors, only a few have addressed the pathogenesis. Experimental animal models may facilitate studies on the early events of the diseases process.¹⁾ Dextran sulfate sodium (DSS)-induced UC is one of the most widely used of these models, and resembles human colitis.^{2,3)} Such models of acute and chronic colitis and its pathology in mice, rats and hamsters have been reported.⁴⁾ However, there have been few reports on the early stage of colitis pathogenesis (within 4 d) after DSS administration, although it has been indicated that intestinal bacteria may be involved, as germ-free mice do not develop colitis when administered DSS.⁵⁾ In addition, it has been reported that intestinal bacteria from mice do not metabolize DSS, suggesting that DSS may exert a direct pathogenic effect.⁶⁾ Accordingly, it is accepted that DSS itself, and not its metabolite(s), is toxic to colonic epithelial cells and causes colitis, although the mechanism involved remains uncertain.⁴⁾ However, the fact that DSS can induce severe colitis in germ-free mice⁷⁾ confuses the issue. Furthermore, as the colitis-inducing ability of DSS in mice is weaker than that in other animals such as hamsters and rats (our unpublished data), there appears to be some difficulty in examining the metabolism of DSS by intestinal bacteria in mice.

The aim of the present study was to clarify the early events of DSS-induced colitis and to examine the metabolism of DSS by intestinal bacteria in rats.

MATERIALS AND METHODS

Chemicals Sodium dextran sulfate 5000 (DSS, molecular weight 5000), hemocult assay kit and solvents of HPLC

grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and Treatment Male Wistar rats (6 weeks old) were purchased from SLC Co. (Hamamatsu, Japan), fed a standard laboratory chow with water *ad libitum*, and maintained for one week. Rats weighing 180–220 g were subjected to the experiments. The rats were maintained in the Laboratory Animal Research Center and the animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at the University of Toyama.

DSS at a concentration of 3% was administered *via* drinking water *ad libitum*. Antibiotics were orally administered from 2 d before DSS administration as follows; 1 mL of a mixed solution containing chloramphenicol (350 mg), nystatin (100 000 units), streptomycin (400 mg), erythromycin (200 mg), and penicillin G (40 000 units) in 20 mL of tap water twice a day, and a mixed solution containing streptomycin (4 g), erythromycin (100 mg), and nystatin (100 000 units) in 1 L of tap water *via* drinking water *ad libitum*, as reported previously.⁸⁾

The gastrointestinal (GI) tracts were removed from the rats under pentobarbital (50 mg/mL/kg) anesthesia. Blood samples were withdrawn from the abdominal vein using capillary tubes heparinized before removal of the GI tracts. The hematocrit value was measured by centrifugation of the tube containing the blood sample.

Hemocult Assay Occult blood testing was carried out using a kit (Wako Pure Chemical Industries, Ltd.). Briefly, a stool or cecal content sample was applied to a thin stick containing guaiac, and then an aliquot of developer (H₂O₂) was dropped onto it for development of a blue color. The color intensity was estimated on a 5-point scale from 1 (negative) to 5 (maximally positive).

Histopathology The intestinal tracts were removed after injection of heparin into the abdominal vein under pentobarbital anesthesia. Each respective part of the GI tract, such as

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the small intestine, cecum, colon and rectum, was rinsed with iced saline to remove the content, immediately fixed in buffered formalin (10%, pH 7.4) for over 24h, embedded in paraffin, sliced into 4 μ m sections and stained with hematoxylin and eosin for light microscopic evaluation.

Measurements Mucosa from intestinal regions such as the cecum, colon and rectum, which had been frozen and thawed before use, was homogenized with 9 volumes of 1.15% KCl to make a 10% homogenate, and then centrifuged. The clear supernatant obtained was measured for growth related oncogene/cytokine-induced neutrophil chemoattractant 1 (GRO/CINC-1) using a rat GRO/CINC-1 measurement kit (Immuno-Biochemical Laboratories Co., Ltd., Tokyo, Japan).

Myeloperoxidase (MPO) activity was measured using the 10% homogenate, as reported previously,⁹⁾ and the 2-thiobarbituric acid (TBA) value was also measured using the 10% homogenate, as reported previously.¹⁰⁾

Incubation of DSS with Cecal Content The incubation mixture (2 mL) consisted of 1 mL of cecal content, 0.4 mL of 5% DSS (sterilized by filtration) and 0.6 mL of distilled water, and then incubated for 24h at 37°C in an anaerobic chamber. The reaction mixture was diluted 3-fold with distilled water and then centrifuged to obtain a clear supernatant. An aliquot (1 mL) of the supernatant was applied to a DE-52 column (1 cm diameter $\times$ 5 cm), which was equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and eluted with the same buffer followed by 0.1 M NaOH. A 2 mL fraction was collected. An aliquot (50 μ L) of each fraction was then determined for total sugar. For this chromatography step, DSS was eluted with 0.1 M NaOH.

On the other hand, another aliquot (1 mL) of the supernatant was applied to a Sephadex G-50 column (1 cm diameter $\times$ 50 cm), which was equilibrated and eluted with 50 mM Tris-HCl buffer (pH 8.0). The fraction collected was 2 mL, and an aliquot (50 μ L) of each fraction was determined for total sugar and sulfate as described below.

Determination of Total Sugar and Sulfate Total sugar was determined using anthrone-H₂SO₄ reagent as reported previously.¹¹⁾ The amount was expressed as the equivalent amount of glucose.

Sulfate was determined using a minor modification of a procedure reported previously.¹²⁾ Briefly, an aliquot of each sample was pyrolyzed in a glass tube by heating directly with a gas burner after lyophilization, and then dissolved in 0.25 mL of deionized water. The solution was mixed with 0.6 mL of barium-containing buffer (5 mL of 2 M acetic acid, 1 mL of 10 mM BaCl₂ and 4 mL of 20 mM NaHCO₃ mixed in 140 mL of ethanol) and 0.3 mL of rhodizonate reagent (a mixture of 5 mg rhodizonate and 100 mg ascorbate dissolved in 20 mL of deionized water, and mixed in 80 mL of ethanol). After being let to stand for 10 min, the reaction mixture was subjected to measurement at $A_{520\text{nm}}$.

Statistical Evaluation The results are expressed as means $\pm$ standard deviation (S.D.) of at least three independent experiments. Statistical analysis was performed using the unpaired Student's *t* test following the *F* test for two groups. Groups were considered significantly different when the *p* value was below 0.05.

RESULTS

Acute Lesions of the Cecal Mucosa Immediately after

DSS Administration When 3% DSS was administered *via* drinking water to Wistar rats, bloody feces and diarrhea were observed even one day after administration. The hemocult scores of feces at 1 d and 3 d after administration were markedly high (scores 4–4.5), in contrast to negative values (score 1) in control rats (Fig. 1). Moreover, blood was detected in the alimentary tracts, mainly in the cecum. The hemocult scores for cecal contents on days 2 and 4 after administration were maximal at 5, in contrast to negative values (score 1) in control rats (Fig. 2), indicating that the blood in feces was derived mainly from the cecal mucosa.

Histopathological analyses confirmed severe lesions of the cecal and colonic mucosae on days 2 and 4 after DSS administration. Severe overall erosion of the cecal epithelium with crypt dilatation, inflammatory acute infiltration, vascular congestion, loss of goblet cells, and crypt abscesses were observed in all of four rats tested (Figs. 3A, a and b, cecal mucosae at 2 and 4 d after administration). However, the mucosae of the small intestine and rectum were not injured (data

Fig. 1. Hemocult Scores for Feces in Rats Administered DSS and in Rats Administered Both Antibiotics and DSS

Feces obtained 1 d (straight columns) and 3 d (dashed columns) after administration of DSS to rats were examined by the hemocult test (scores 1–5). The columns show the scores for feces in rats after administration of DSS alone (DSS), without administration (control), administration of antibiotics from 2 d before DSS administration (antibiotics+DSS), and administration of antibiotics alone (antibiotics). Each column represents the mean $\pm$ S.D. of four different experiments. ***p* < 0.01 (DSS vs. antibiotics+DSS).

Fig. 2. Hemocult Scores for Cecal Contents in Rats Administered DSS and in Rats Administered Both Antibiotics and DSS

Cecal contents obtained 2 d (straight columns) and 4 d (dashed columns) after administration of DSS to rats were examined by the hemocult test (scores 1–5). The columns are the same as those in Fig. 1. Each column represents the mean $\pm$ S.D. of four different experiments. ***p* < 0.01 (DSS vs. antibiotics+DSS).

Fig. 3. Photomicrography of Cecal Sections from Rats Administered DSS and from Rats Administered Both Antibiotics and DSS

Rats were administered DSS alone (A, DSS), were administered antibiotics from 2d before DSS administration (B, antibiotics+DSS), or were not treated (C, control). The intestine was removed 2d and 4d after administration of DSS, and then processed as described in Materials and Methods. 3A, a: DSS, 2d; b: DSS, 4d; 3B, c: antibiotics+DSS, 2d; d: antibiotics+DSS, 4d; 3C, e: control; ($\times 100$).

not shown).

On the other hand, values of inflammatory markers, such as GRO/CINC-1 and MPO activity, in the cecal and colonic mucosae of rats at 4 d after administration were almost the same as those of control rats, although the values of these markers after day 6 of administration were increased to significantly higher levels than those of the control (data not shown). Hematocrit values were also decreased after the day 6. Moreover, the TBA values of the mucosa were increased, and colon length was shortened after day 10 of administration. These results indicated that DSS initially injured the cecal mucosa, and then inflammatory colitis spread throughout the lower part of the alimentary canal, including the colon and rectum.

Repression of DSS-Induced Lesions by Administration of Antibiotics When antibiotics, which kill most of the intestinal bacteria,⁸⁾ were administered from 2 d before DSS administration, bloody feces were not observed and the hemocult scores of the feces on days 1 and 3 after administration of DSS were significantly lower than those of rats administered DSS alone (Fig. 1). In addition, the hemocult scores for cecal contents on days 2 and 4 after administration were also significantly lower than those of rats administered DSS alone (Fig. 2). Moreover, histopathological analyses also showed that administration of antibiotics repressed the DSS-induced injury of the cecal and colonic mucosa in all of four rats tested. The cecal mucosa on days 2 and 4 after administration of DSS with antibiotics (Figs. 3B, c and d) was similar to those of control rats (Figs. 3C, e) and rats administered antibiotics.

Metabolism of DSS by Rat Cecal Content The metabolism of DSS by rat cecal content was investigated *in vitro*. When cecal contents from conventional rats were incubated with DSS at 37°C in an anaerobic chamber, the sugar content of fractions corresponding to DSS was decreased to almost half after 24 h incubation, as judged by DE-52 column chromatography (data not shown). Also, when the reaction mixture was applied to a Sephadex G-50 column, the peak of the high-molecular-weight fractions (Frs. 6–12) was broad, and spread to low-molecular-weight fractions (Fig. 4) in comparison with the sharp peak (Fr. 8, arrow) of the original DSS, indicating that DSS had been degraded. In addition, the sulfate content of the high-molecular-weight fractions was decreased to about 40% of the sugar (glucose equivalent) content to yield free sulfate ions (Fr. 18, arrow), compared with the original DSS, which exhibited a coincident peak showing an equal ratio of sulfate and sugar. These results indicate that DSS was initially desulfated.

Moreover, the cecal contents of rats administered DSS for 2 d were examined for *in vivo* metabolism of DSS. Free sulfate ions ($9.09 \pm 4.34 \mu\text{mol/mL}$ of cecal content) together with low-molecular-weight sugars in only small amounts were detected in the cecal contents of rats administered DSS, but not in those of control rats, as judged by Sephadex G-50 column chromatography. These results suggest that DSS was metabolized in rat cecum, similar to the *in vitro* metabolic pattern.

Repression of DSS Metabolism by Administration of Antibiotics The cecal contents from rats that were administered antibiotics for 2 d did not metabolize DSS at all even after 24 h of incubation, as judged by both DE-52 and Sephadex G-50 column chromatography (data not shown), indicating participation of intestinal bacteria in DSS metabolism. Thus, the sugar and sulfate contents of the high-molecule-weight

Fig. 4. Sephadex G-50 Column Chromatography of an Incubation Mixture of DSS with Cecal Content from a Conventional Rat

DSS was incubated with the cecal content from a conventional rat, and the reaction mixture was applied to a Sephadex G-50 column. Sulfate (solid line) and total sugar (glucose equivalent, dashed line) are expressed as amounts in the fraction tube.

Fig. 5. Total Sugar and Sulfate Content of the DSS Moiety after Incubation with Cecal Content

DSS was incubated with cecal contents from conventional rats (left side) and rats administered antibiotics for 2 d (right side). The reaction mixtures were then applied to a Sephadex G-50 column (Fig. 5). Amounts of total sugar (glucose equivalent) and sulfate in the high-molecular-weight fractions corresponding to DSS, which were eluted from the columns, were summed, and are shown as straight and dashed columns, respectively. Each column represents the mean $\pm$ S.D. of three different experiments. ** $p < 0.01$.

fractions (Frs. 6–12) in the latter case for the conventional rats were significantly lower, 78.6% and 35.0% respectively, than those for rats administered antibiotics (Fig. 5), indicating that the carbohydrate moiety was subsequently degraded after desulfation.

DISCUSSION

In the present study, DSS-induced UC was investigated using a widely employed animal model in which the pathologic features are similar to those of human IBDs. Although experimental animal models may allow studies of early events in the pathogenesis of these diseases,¹⁾ only a few studies have investigated this issue, despite numerous reports on pathologic changes in the colon and rectum.

Bloody feces were observed in rats from as early as 1 d after administration, and the hemocult scores for feces at this point were markedly higher than those in control rats (Fig. 1). In addition, blood was observed in the cecum, and the hemocult scores for the cecal contents were maximal (score 5) 2 d after DSS administration (Fig. 2). Furthermore, severe lesions

of the cecal mucosa 2 d after administration confirmed by histopathological examination (Fig. 3A), although many previous studies have reported lesions of the colonic and rectal mucosae after a longer period (4 d or more) of DSS administration.⁴⁾ Severe mucosal erosion with necrotic foci and massive neutrophil infiltration in the mucosa and submucosa were observed (Fig. 3A). These lesions mimicked the feature of Mats grade 5, which is severest histological grading of human UC. Moreover, administration of antibiotics prevented both bleeding and cecal mucosal lesions induced by DSS (Figs. 1, 2, 3B). These results suggest the importance of the cecum and the participation of intestinal bacteria in the pathogenesis of DSS-induced colitis. However, the relationship between the pathogenesis of DSS-induced colitis and the intestinal microbiota has not been clarified.

DSS is not degraded *in vitro* by the mouse intestinal microbiota,⁶⁾ suggesting a negligible contribution of the intestinal microbiota to colitis pathogenesis through DSS degradation. However, in the present study, DSS was degraded completely to yield sulfate ions, both *in vitro* (Figs. 4, 5) and *in vivo*. Thus, DSS was initially desulfated, and then the residual carbohydrate moiety was degraded. The discrepancies among previous studies may be due to species differences, *i.e.*, between mouse and rat, because DSS induces colitis far less readily in mouse than in rat (our unpublished data) and marked differences exist in bacterial composition and metabolism of the gut microbiota of different species of animal.¹³⁾ This also suggests a relationship between DSS metabolism and induction of colitis. If this is indeed the case, which specific metabolite of DSS induces colitis? One possibility is the sulfate ion, which was shown to be produced efficiently from DSS by rat intestinal bacteria (Figs. 4, 5). Sulfate can be reduced to give sulfite by intestinal bacteria from healthy subjects and patients with UC,¹⁴⁾ and this shows potent toxicity that may be implicated in the pathogenesis of UC.¹⁵⁾ This hypothesis is supported by a report that the activity of rhodanese, which directly or indirectly detoxifies sulfide, is elevated in red blood cells of mice with DSS-induced colitis.¹⁶⁾ However, our preliminary data (unpublished) indicate that oral administration of sodium sulfate to rats does not induce colitis, because the sulfate administered does not reach the lower intestine due to its absorption in the upper intestine. Further research is expected to yield more information about the pathogenesis of DSS-induced colitis.

In summary, we have shown that when DSS is administered to rats, the cecal mucosa is severely injured, together with production of bloody feces and blood in the cecum, within 2 d after administration. However, these severe lesions of the cecal mucosa are repressed by administration of antibiotics. On the other hand, DSS is metabolized by the cecal content, being first desulfated and then degraded completely, whereas this is not the case for cecal content from rats administered antibiotics. These results indicate that the initial step of DSS-induced UC is lesioning of the cecal mucosa, which is related to metabolism of DSS by intestinal bacteria.

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Conflict of Interest The authors declare no conflict of interest.

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