0031-3998/02/5301-0143
PEDIATRIC RESEARCH

Vol. 53, No. 1, 2003
Printed in U.S.A.

Dextran Sulfate Sodium–Induced Inflammation Is Enhanced by Intestinal Epithelial Cell Chemokine Expression in Mice

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ABSTRACT

Dextran sulfate sodium (DSS) induces an inflammatory bowel disease–like colitis in animals. To determine the contribution of epithelium to inflammation in the intestine, we examined the effects of DSS in transgenic mice that specifically secrete macrophage inflammatory protein-2 (MIP-2) from the intestinal epithelium. We first confirmed the production of MIP-2 from intestinal epithelial cells by Western blots in transgenic mice. MIP-2 transgenic mice were therefore an appropriate model to examine the role of epithelial cell chemokines in an inflammatory state induced by DSS. We then examined the neutrophil migration into the intestine and the effect of DSS on this migration by myeloperoxidase staining. There was an increase of myeloperoxidase-positive neutrophils in the intestine from wild-type and transgenic mice after the DSS treatment. Furthermore, the increase of neutrophils under stimulation with DSS was confirmed quantitatively by measuring specific tissue myeloperoxidase activities. It was significantly greater in DSS-treated MIP-2 transgenic mice than in wild-type mice in both the small intestine and colon. These results suggest that the inflammatory effects of DSS on both small intestine and colon are enhanced by MIP-2 secreted by epithelial cells in the transgenic mice. In conclusion, intestinal epithelial cells can act in concert with other inflammatory stimuli in maintaining inflammation. **(*Pediatr Res* 53: 143–147, 2003)**

Abbreviations

CMF-HBSS, Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution
DSS, dextran sulfate sodium
Fabpi, fatty acid binding protein of the intestine
IBD, inflammatory bowel disease
IEL, intraepithelial lymphocyte
MIP-2, macrophage inflammatory protein-2
MPO, myeloperoxidase

The intestinal epithelium regulates the absorption of nutrients and serves as a barrier to the external environment. Because the enterocyte is ideally placed to signal changes in the intestinal milieu through the expression of immunologically active proteins, it is also possible that epithelial cells are capable of signaling changes in the intestinal lumen to immune cells in the lamina propria or Peyer's patches (1, 2). Candidates for signaling include antigen presentation to mucosal T cells (3–6) and chemokine secretion (7–14). *In vitro* experiments have repeatedly demonstrated that epithelial cell lines respond to bacterial pathogens by secreting chemokines (9–12). Moreover, changes in normal intestinal contents vary the expression of chemokines by epithelial cells (4, 13, 14). For example, butyrate, a short-chain fatty acid derived from the fermentation of nonabsorbed carbohydrate by intestinal bacteria, regulates the expression of MIP-2 in mouse and IL-8 in human intestinal epithelial cells. However, the contribution of the epithelium to inflammation established in the intestine is not clear. It is possible that when children with Crohn's disease receive enteral feedings (15), the changes in the intestinal lumen are detected by the epithelium and relayed to the mucosal immune system (16). To test the hypothesis that epithelial cell signaling influences the degree of inflammation established in the intestine, we compared the effects of DSS in normal mice with mice whose intestinal epithelial cells of the small intestine and proximal colon secrete MIP-2. The exact mechanism whereby DSS causes inflammation is not fully elucidated; however, it

Received September 13, 2001; accepted June 20, 2002.
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Supported by grants from NIH (AI43472, DK47753), the Medical Research Council, the Joint Research Board of St Bartholomew's Hospital, The Mercers' Company, and the Uehara Memorial Foundation, Tokyo, Japan.

DOI: 10.1203/01.PDR.0000041519.16507.06

induces reproducible colitis in rodents (17, 18) and it is an established model of IBD.

MIP-2 is a member of the α-chemokine family, which includes human IL-8 (which is not expressed in the mouse), human GROα, rat KC/CINC, and murine KC. As with IL-8 in humans, MIP-2 is chemotactic for neutrophils and induces a localized neutrophil infiltration when injected into the hind footpads of mice (19–21). Therefore, MIP-2 acts in the mouse in a similar manner to IL-8 in humans. MIP-2 is only expressed in intestinal epithelial cells after stimulation with lipopolysaccharide or proinflammatory cytokines (13, 14). Since normal intestinal epithelial cells do not express MIP-2, inducing MIP-2 expression in the epithelium using transgenic techniques enabled us to study the effect of chemokine signaling by epithelial cells in the mucosal immune system *in vivo*. MIP-2 transgenic mice were generated by using an epithelial cell–specific promoter from the gene encoding Fabpi (22–24). Because the Fabpi promoter is specifically active only in the intestinal epithelium, MIP-2 is continuously expressed from intestinal epithelial cells in these mice but not in wild-type mice (25).

In this article, we investigate the interaction of intestinal epithelial cells with another chemical stimulus by administering DSS into both normal (wild-type) and MIP-2 transgenic mice, and discuss the role of intestinal epithelial cells in the mucosal inflammation.

METHODS

Mice. MIP-2 transgenic mice and wild-type mice aged 3 to 6 mo were used. MIP-2 transgenic mice were established using the plasmid containing the Fabpi promoter linked to the MIP-2 gene and the SV40 intron and polyadenylation site (Fig. 1). Because Fabpi is specifically expressed by intestinal epithelial cells, these mice express MIP-2 mRNA and produce MIP-2 from the intestinal epithelium without any stimulation (25). MIP-2 transgenic mice develop normally, with no diarrhea, and reveal no significant differences in body weight compared with wild-type mice. Some of both wild-type and transgenic mice were given 2.5% DSS (Sigma Chemical Co., St. Louis, MO, U.S.A.) in drinking water for 10 d to induce intestinal inflammation (17, 18). Experiments were approved by the Subcommittee on Research Animal Care of Massachusetts General Hospital, Boston MA, U.S.A., and by the Animals (scientific procedures) Act 1986, Home Office, London, U.K.

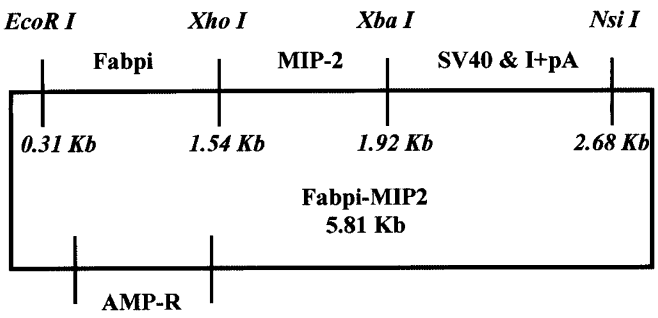

Figure 1. Generation of MIP-2 transgenic mice. Diagrammatic representation of the plasmid containing the Fabpi promoter linked to the MIP-2 gene and the SV40 intron and polyadenylation site. MIP-2 cDNA was derived from a mouse MIP-2 sequence using PCR with primers synthesized with an *Xho*I site and an *Xba*I site.

Epithelial cell preparations. Each intestinal sample was removed and flushed with CMF-HBSS (Sigma Chemical Co.), and all Peyer's patches were removed, before isolation of epithelial cells. The intestine was then opened longitudinally, washed gently with CMF-HBSS, and cut laterally into small pieces. Each segment was incubated in CMF-HBSS containing 1 mM EDTA and stirred for 20 min four times to remove epithelium (26). Cell suspension was filtered through nylon mesh and centrifuged. The cell pellet, consisting of epithelial cells and IEL, was suspended in RPMI 1640 medium (Sigma Chemical Co.) containing 10% FCS, 10 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin. Then IEL were removed from the epithelial cell suspension using a 45% to 70% discontinuous Percoll (Sigma Chemical Co.) gradient as previously described (26).

Cytoplasmic MIP-2 extraction and MIP-2 Western blot analysis. Epithelial cell membranes were ruptured with hypotonic buffer, containing 10 mM HEPES, 10 mM KCl, and 1.5 mM $MgCl_2$, and the resulting cytoplasmic protein was extracted by centrifugation at 3300 $\times$ *g* for 15 min at 4°C. MIP-2 was detected on Western blot analysis as previously described (13). Samples were mixed 1:2 in 2$\times$ treatment buffer (0.5 M Tris-HCl/4% SDS/20% glycerol/10% 2-mercaptoethanol, pH 6.8) and heated in boiling water for 3 min. Samples were loaded into an SDS/3.5% polyacrylamide gel stacker over an SDS/15% polyacrylamide gel, and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA, U.S.A.) for 90 min at 400 mA at 4°C. After blocking with 5% nonfat dry milk, nitrocellulose membranes were incubated with a goat anti-mouse MIP-2 antibody (Sigma Chemical Co.) at a 1:2000 dilution and stored overnight at 4°C. After washing with PBS, membranes were incubated with a 1:8000 dilution of horseradish peroxidase–labeled rabbit anti-goat IgG (Sigma Chemical Co.). Epichemiluminescence detection reagents (Amersham International, Buckinghamshire, U.K.) were applied according to the manufacturer's instructions. Mouse recombinant MIP-2 (Sigma Chemical Co.) was used as a positive control.

Histologic analysis. Twenty-five milliliters of CMF-HBSS was perfused transcardially to flush all blood components from the intestinal vasculature. Intestine was then removed and washed gently with CMF-HBSS and snap-frozen in liquid N_2. The neutrophil infiltration of the intestinal mucosa was examined by staining frozen sections (4 μm) for MPO using the Hanker Yates reaction (27). Sections were incubated with Hanker Yates reagent (Sigma Chemical Co.), 1 mg/mL in 10 mL of 0.1 M Tris buffer (pH 7.6) containing 1 μL/mL of 3% H_2O_2 for 30 min and counterstained with hematoxylin before examination by light microscopy.

Total MPO activity. For measurement of functional MPO activity, tissue specimens were weighed and homogenized in 5 mL of 50 mM phosphate buffer (pH 6.0) at 4°C, and centrifuged at 30,000 $\times$ *g* for 30 min at 4°C. The pellet was extracted with 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co.) in 50 mM phosphate buffer (pH 6.0) at 25°C

(28). Samples were frozen on dry ice and sonicated. These were then centrifuged at 30,000 × *g* for 30 min. Supernatants were reacted with o-dianisidine dihydrochloride (Sigma Chemical Co.) containing 1 μL/mL of 3% H_2O_2, and the MPO activity was assayed spectrophotometrically. Results are expressed per unit weight of intestine.

The thickness of the small intestine is greater than that of the colon, owing mainly to greater amounts of muscle and connective tissue. Expression of MPO activity as a proportion of unit weight of whole tissue appears, therefore, to be greater in colon samples than in small intestine. Nevertheless, this does not invalidate comparisons of similar organs between wild-type and transgenic mice. Each experiment was repeated three times with age-matched mice between 3 and 6 mo of age. Treatment groups consisted of eight mice.

Statistics. All data were analyzed by performing two-tailed, unpaired *t* tests. A $p < 0.05$ was considered significant.

RESULTS

Fabpi gene induces MIP-2 expression in epithelial cells in transgenic mice. Epithelial cells derived from wild-type mice accumulated no MIP-2 mRNA by either Northern blots or reverse transcriptase-PCR. However, both methods detected MIP-2 transcripts in the intestinal epithelium of MIP-2 transgenic mice (25). To confirm the production of MIP-2 from intestinal epithelial cells, epithelial cells were isolated and protein was extracted. The production of MIP-2 from intestinal epithelial cells was not detected extracellularly. However, MIP-2 protein was present in cytoplasmic lysates of epithelial cells from each MIP-2 transgenic mouse, whereas there was no MIP-2 observed in wild-type mice examined by Western blots (Fig. 2).

Production of MIP-2 by epithelial cells increases the inflammatory response caused by DSS. Because MIP-2 is known as a chemoattractant for neutrophils (17, 18), we examined whether the MIP-2 secreted by intestinal epithelial cells increased neutrophil recruitment *in vivo*. As previously demonstrated, there were few MPO-positive neutrophils in the small intestine and colon in wild-type mice. However, MPO-positive neutrophils were increased in the lamina propria of MIP-2 transgenic mice (25), both in the small intestine (Fig. 3, upper panels) and colon (Fig. 4, upper panels), when examined histologically or by quantitating total MPO activity per unit weight (Fig. 5).

DSS administered in drinking water is known to reproducibly induce mild intestinal inflammation with ulceration in mice (17, 18). To study whether epithelial expression of a chemokine increases neutrophil recruitment under conditions in which intestinal inflammation is triggered by other stimuli, DSS was administered in drinking water to wild-type and MIP-2 transgenic mice. A concentration of 2.5% DSS was chosen because it did not cause bloody diarrhea either in wild-type or MIP-2 transgenic mice, because blood can enhance the total MPO activity irregularly. This concentration was sufficient to induce neutrophil invasion into the intestine.

In wild-type mice, DSS induced a mild increase of neutrophil migration, observed in the intestine by histologic analysis. However, secretion by the epithelial cells of MIP-2 in transgenic mice reproducibly increased the intestinal inflammation induced by DSS treatment both in the small intestine (Fig. 3, lower panels) and the colon (Fig. 4, lower panels).

These findings were confirmed quantitatively by measuring total MPO activity in the whole small intestine and colon. The total MPO activity was significantly elevated after DSS treat-

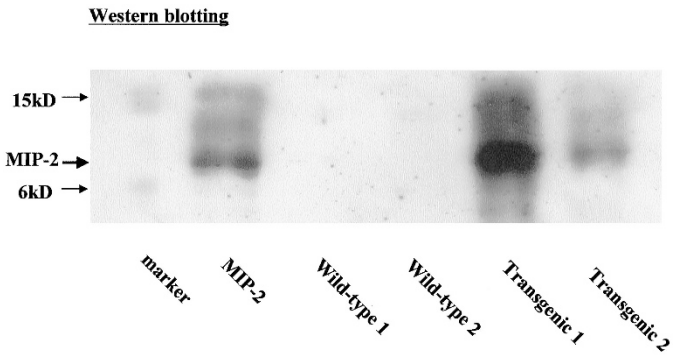

Figure 2. Cytoplasmic MIP-2 was detected by Western blot analysis. Cytoplasmic protein was extracted from wild-type and MIP-2 transgenic mice. MIP-2 was detected by a goat anti-mouse MIP-2 antibody. Mouse recombinant MIP-2 was used as a positive control. Results are representative of three independent experiments.

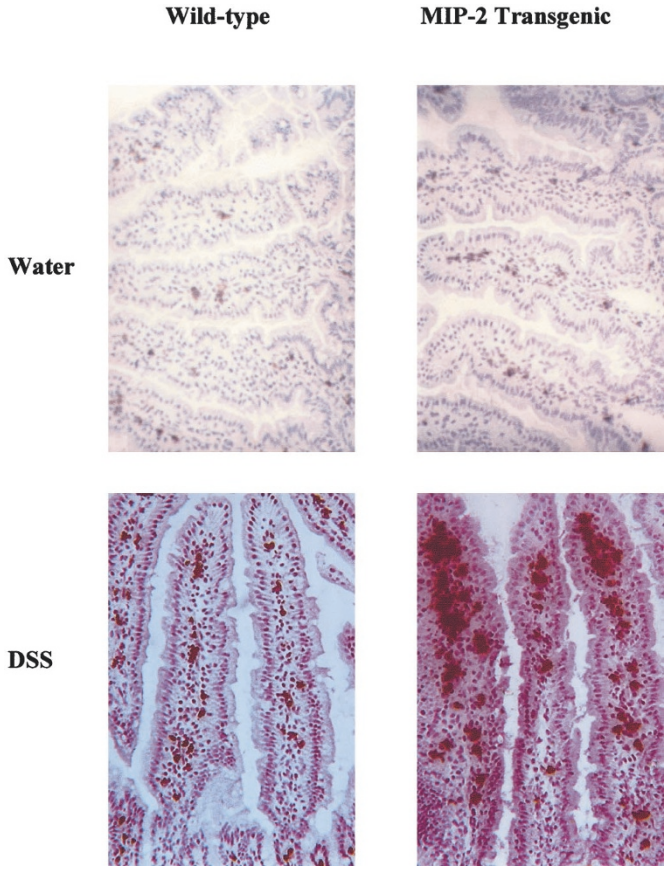

Figure 3. Neutrophil recruitment in the small intestine from wild-type and MIP-2 transgenic mice with or without 2.5% DSS treatment. Neutrophil recruitment was greater in MIP-2 transgenic mice than in wild-type mice after DSS treatment. Neutrophils were stained by MPO using the Hanker Yates reaction. Magnification ×100.

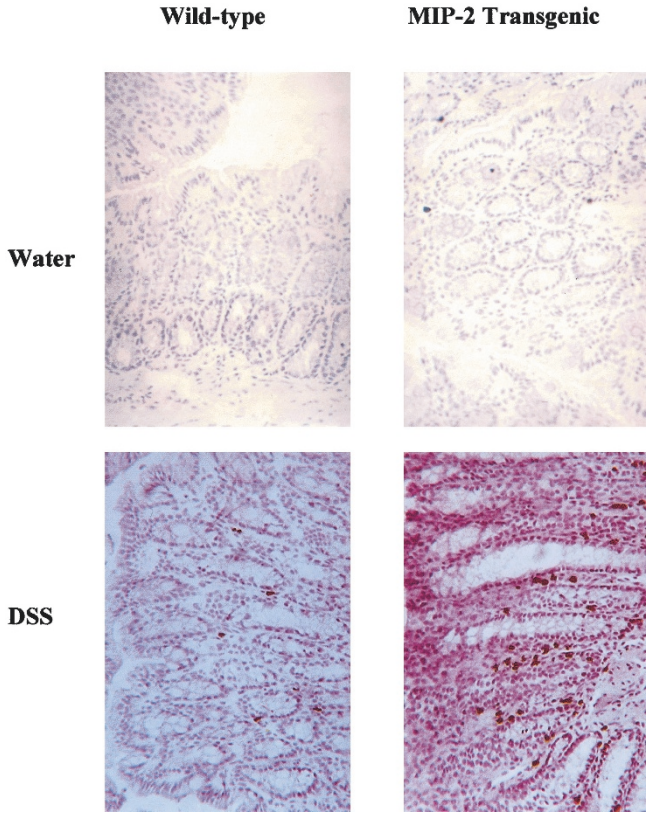

Figure 4. Neutrophil recruitment in the colon from wild-type and MIP-2 transgenic mice with or without 2.5% DSS treatment. Neutrophil recruitment was greater in MIP-2 transgenic mice than in wild-type mice after DSS treatment. Neutrophils in the colon were stained by MPO using the Hanker Yates reaction. Magnification ×100.

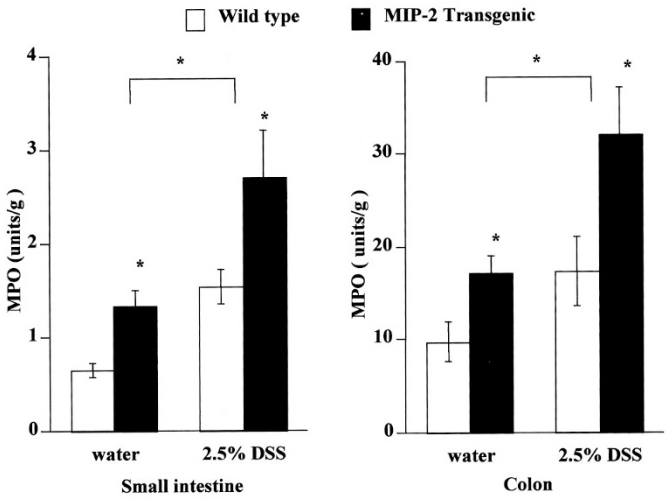

Figure 5. Neutrophil recruitment was much greater in MIP-2 transgenic mice than in wild-type mice both in the small intestine and colon after 2.5% DSS treatment. Neutrophil recruitment was demonstrated by total MPO activity. MPO was extracted from the whole small intestine and colon with 0.5% hexadecyltrimethylammonium bromide. Open columns show total MPO activity per unit weight of tissue derived from wild-type mice, and closed columns show those of MIP-2 transgenic mice. Data are presented as the mean ± SD of eight animals for each group. *$p < 0.01$.

ment in the wild-type mice both in the small intestine and colon ($p < 0.01$). Thus, the elevation of total MPO activity after DSS treatment was much greater in the MIP-2 transgenic mice both in the small intestine and colon ($p < 0.01$; Fig. 5).

DISCUSSION

The increased infiltration of neutrophils and lymphocytes into the gastrointestinal tract is a hallmark of both ulcerative colitis and Crohn's disease. However, the contribution of epithelial cell chemokine secretion to the observed inflammatory changes has never been studied in a model of IBD. In this study we used the epithelial-specific expression of MIP-2 in transgenic mice to examine the hypothesis that chemokines secreted by the epithelium combine with the inflammatory processes generated in IBD. We used DSS treatment as a model of IBD, as it has been well validated and is reproducible. We have shown that the expression of even one chemokine by the epithelial cell doubled the neutrophil recruitment into the lamina propria of both colon and small intestine, suggesting that the epithelial cell chemokine secretion may multiply the effect of other inflammatory stimuli in the intestine.

Although the action of DSS is not completely understood, it appears to be phagocytosed by macrophages. These macrophages have enlarged lysosomes and contain a polysaccharide sulfate that may reduce phagocytic activity (17). In addition, DSS causes a change in the intestinal microflora, and particularly an increase in the number of Gram-negative anaerobes (17). DSS may also be cytotoxic to epithelial cells and IEL, and may interfere with the normal interaction between intestinal lymphocytes, epithelial cells, and extracellular matrix (29). Treatment with DSS is therefore a good animal model for intestinal inflammation. Although DSS is reported to have its main site of action in the large intestine (probably because of the distribution of macrophages), we found significant neutrophil recruitment in the small intestine after DSS treatment by measuring total MPO activity (Fig. 5). The lack of reports of DSS action in inducing small bowel inflammation is not easily explained. However, there have been no reports in which neutrophils have been examined by MPO activity with flushing of all blood components from the vasculature as a pretreatment. Thus the finding of the greatly increased neutrophil recruitment in MIP-2 transgenic mice, which may produce more MIP-2 in the small intestine than do wild-type mice (Fig. 5), suggests that chemokine production from the epithelium is an important mechanism of DSS-induced mucosal inflammation. DSS may therefore stimulate intestinal epithelial cells and enhance the chemokine secretion to further increase neutrophil invasion in these mice.

The present study provides evidence that down-regulating epithelial chemokine secretion would result in reduced inflammation, even when other mechanisms in the intestine have initiated it. Any treatment regimen that decreases chemokine secretion in the epithelial cell is likely to reduce inflammation in IBD. It is possible that certain antiinflammatory drugs or dietary regimens such as enteral diets act in this way as part of their mechanism of action (2).

CONCLUSION

In summary, this study supports the hypothesis that epithelial cell chemokine secretion alters the amount of inflammation

in the intestine, even when this is induced by inflammatory processes in the intestinal lumen.

Acknowledgments. The authors thank Drs. E. Schmidt and P. Leder for the JvSV plasmid, and Dr. J. Gordon for Fabpi promoter.

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