Constitutive expression of MMP9 in intestinal epithelium worsens murine acute colitis and is associated with increased levels of proinflammatory cytokine Kc

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Inflammatory bowel disease (IBD), which includes ulcerative colitis and Crohn’s disease, is a chronic inflammatory disease associated with an increased risk for colon cancer. Matrix metalloproteinases (MMPs) are the predominant proteases expressed in the gut mucosa during active IBD. Our laboratory has previously demonstrated that epithelial-derived MMP9 is absent in normal colonic tissue but is upregulated during IBD. In this study MMP9 transgenic mice (Tg-villin-MMP9) are generated specifically to overexpress MMP9 in intestinal epithelium to examine the role and underlying mechanism by which it modulates the pathogenesis of acute colitis. Dextran sodium sulfate (3% DSS)- and Salmonella typhimurium (S.T.)-induced colitis models were used to study gut inflammation in Tg-villin-MMP9 and wild-type littermates (WT). Colonic tissue was analyzed via Western blot, histology, myeloperoxidase (MPO) assay, and quantitative PCR. Tg-villin-MMP9 mice expressed significantly increased MMP9 mRNA and protein expression at basal level. There was a significant decrease in the goblet cells, but a significant increase in proliferation and apoptosis were observed among Tg-villin-MMP9 mice compared with WT mice. There was also a significant increase in the proinflammatory chemokine Kc among Tg-villin-MMP9 compared with WT mice. Tg-villin-MMP9 exhibited a severe inflammatory response than WT mice in both DSS- and S.T.-induced colitis models as evident by greater weight loss and higher clinical score, histological score, and MPO activity, which correlated with relative levels of Kc mRNA. MMP9 expressed by intestinal epithelial cells mediates inflammation in colitis with simultaneous increase in proinflammatory cytokine Kc.

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn’s disease (CD), is a chronic inflammatory disease that affects the entire gastrointestinal (GI) tract and the colonic mucosa and is associated with an increased risk for colon cancer. IBD is characterized by relapses (acute flare) and remissions, which are related to genetic basis and involve immune-mediated tissue injury followed by repair. Recent studies have shown that cytokines play a key role in regulating IBD (9, 40). There is increased incidence of IBD in urban areas of industrialized nations (29, 42). Matrix metalloproteinases (MMPs) are a group of zinc-dependent enzymes with proteolytic activity against extracellular matrix (ECM) proteins and are also known to play an important role for normal tissue remodeling (47). MMPs have also been shown to be involved in several pathological conditions such as IBD, tumor invasion, and colorectal cancer (10, 19, 38). Furthermore, MMPs play a crucial role in regulating the physiology and pathophysiology of IBD by modulating leukocyte accumulation into inflamed tissue and cytokine production from inflammatory and epithelial cells (36). MMPs can be classified into four major subgroups depending on substrate specificity, amino acid similarity, and identifiable sequence modules (39). It has been reported previously by us and other groups that MMP9, which is one of the two known gelatinases, is predominantly upregulated in the animal models of colitis and plays a major role in driving gut inflammation in both infectious and chemical-induced colitis (5, 6, 12, 25, 36). Our laboratory has also shown that such MMP9 driven inflammation was largely mediated by MMP9 expressed by epithelial cells in response to the inflammatory challenge (12). Furthermore, MMP9 activity is correlated with active inflammation associated with fistula formation (43, 48). In addition, MMP9 tissue and serum levels correlate with disease activity in UC (1, 28, 35).

In this study we utilized MMP9 transgenic mice (Tg-villin-MMP9) generated specifically to express MMP9 in intestinal epithelium to examine the role and underlying mechanism by which MMP9 modulates the pathogenesis of both infectious and chemical-induced colitis.

MATERIALS AND METHODS

Animal model. All animal procedures were in compliance with the Guide for the Care of Use of Laboratory Animals from the US Public Health Service and were approved by the Animal Care Committee of Georgia State University. In collaboration with the Transgenic Mouse and Gene Targeting Core Facility (Emory University), we generated the homozygous Tg-villin-MMP9 mouse model in C57/B6 mice and their wild-type (WT) littermates used for the study. We received heterozygous Tg-villin-MMP9 mice from the core facility and crossed them to generate homozygous. WT littermates obtained during the generation of the homozygous strain were used as WT controls throughout the study. Six- to 8-week-old Tg-villin-MMP9 and WT mice were used for the study. The mice were kept on a 12-h dark-light cycle and given access to pelleted, nonpurified diet and tap water under controlled temperatures (25 ± 2°C).

In vivo permeability. In vivo permeability assay to assess barrier function was performed by using a FITC-labeled dextran method as described previously (23). Briefly, 8- to 10-week-old age- and sex-matched Tg-villin-MMP9 and WT mice were used. Food and water were withdrawn for 4 h and mice were gavaged with permeability tracer (60 mg/100 g body wt of FITC-labeled dextran, mol wt 4,000;
Sigma). Serum was collected retroorbitally and fluorescence intensity of each sample was measured (excitation, 492 nm; emission, 525 nm; Cytofluor 2300; Millipore, Waters Chromatography, Bedford, MA), and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran. Permeability was calculated by linear regression of sample fluorescence.

**Induction and mortality of DSS colitis.** Age- and sex-matched Tg-villin-MMP9 and WT mice were given an oral administration of dextran sodium sulfate (DSS; ICN Biomedicals, Aurora, OH) at 3% (wt/vol) in tap water ad libitum for 6 days. Age- and sex-matched Tg-villin-MMP9 and WT mice supplied with normal drinking water served as controls. As described previously (25), mice were evaluated daily for changes in body weight and onset of clinical symptoms of colitis. The colon length and weight were measured and descending colon tissue sections were obtained for further study. For mortality assessment mice were given DSS water for 6 days and then normal drinking water for an additional 8 days. Mice were euthanized during mortality assessment if they lost ≥20% body weight, as per approved animal protocol guidelines to meet the end point criteria.

**Clinical score.** On a daily basis the mice were evaluated for a change in body weight, stool consistency, and the presence of occult or gross blood by a guaic test (Hemoccult Sensa; Beckman Coulter, Fullerton,

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Fig. 1. Characterization of transgenic mice with intestinal epithelial cell-specific overexpression of MMP9. As described in MATERIALS AND METHODS, mRNA and Western blot lysates were extracted from colonic mucosal stripping of 6- to 8-wk-old Tg-villin-MMP9 mice and their wild-type (WT) littermates. **A:** quantification of mRNA levels for MMP9 using real-time PCR. **B:** Western blot of proteins (30 μg/lane) from the mucosal stripping of the colons were probed with anti-MMP9. Western blots are quantified by scanning densitometry. **C:** hematoxylin and eosin (H&E) staining of Swiss rolls of colon showing crypt architecture and muscularis mucosa layer. **D:** Alcian blue-periodic acid Schiff staining of Swiss rolls of colon showing the goblet cells and mucin granules. **E:** bar graph showing the percentage of the number of goblet cells per crypt (12 crypts/mouse were counted). **F:** bar graph showing the fluorescence of retroorbitally obtained serum and measured as mg FITC·μg protein⁻¹·h⁻¹. **G:** Swiss rolls of colons of mice were processed for Muc-2 staining (×20 magnification). Each bar represents means ± SE (n = 6/group). *P < 0.05.
CA). The clinical score was determined on the basis of weight loss, stool consistency, and fecal blood as described by Cooper et al. (14).

**Hematoxylin and eosin staining and histological score.** The descending colon of mice induced with DSS colitis and cecum for S.T.-induced colitis were collected as described previously (25) and were analyzed for histological score based on three parameters: crypt damage, infiltration of neutrophils, and foci of ulceration (14).

**Immunohistochemistry.** Colonic tissues obtained as described previously (24) were embedded in paraffin and analyzed immunohistochemically for Ki67 and Muc-2.

**TUNEL stain.** As described previously (24), terminal deoxyuridine nick-end labeling (TUNEL) was used to identify apoptotic cells.

**Isolation of mRNA and quantitative PCR.** Total RNA was isolated from mucosal stripping of mouse colons by using the RNeasy Kit (Qiagen, Valencia, CA) as per manufacturer’s instruction. Total RNA was then reverse transcribed by use of the RevertAid first-strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). A total of 10 ng of reverse-transcribed complementary DNA was amplified by quantitative real-time PCR with 10 μmol/l TNF-α, IFN-γ, Kc, and IL-6 specific primers (18) and Fermentas Maxima SYBR Green qPCR master mix (Fermentas) under the following PCR conditions: initial denaturation of 1 cycle at 95°C for 3 min, followed by amplification at 95°C for 15 s, 60°C for 1 min for 40 cycles using the real-time RealPlex 4S sequence detection system (Eppendorf, Hauppauge, NY). Expression level of 36B4 was used as an internal control. Raw cycle threshold values (Ct values) obtained for any of the above mention cytokines were deducted from the Ct value obtained for internal 36B4 transcript levels. For graphical representation of quantitative PCR data, the CT was calculated as follows:

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\Delta \Delta CT = (CT_{target} - CT_{36B4})_{Tg-villin-MMP9} - (CT_{target} - CT_{36B4})_{WT},
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where “target” refers to the gene of interest, with the final graphical data derived from $2^{-\Delta \Delta CT}$.

**MPO activity in the colon.** To quantify neutrophil infiltration into the colon myeloperoxidase (MPO) activity was measured as described previously (23).

**Protein extraction and Western blot analysis.** Colonic tissues were obtained as described previously (23) and were subsequently homog-
enzized and extracted with cell lysis buffer and electrophoresed in 10% SDS-PAGE gels. The membranes were incubated overnight at 4°C with antibodies for MMP9 (Abcam, Cambridge, MA). The MMP9 membrane was then washed with Tris-NaCl-Tween 20 and incubated with a goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Bio-Rad, Hercules, CA) for 1 h and the NF-κB membrane was incubated with goat anti-mouse immunoglobulin G horseradish peroxidase conjugate (Bio-Rad). The membranes were then developed with Western Lightning Chemiluminescence Reagent plus (GE Healthcare, Piscataway, NJ). Quantification was performed by image analysis.

**Organ culture ELISA.** Proinflammatory cytokine Kc was measured by ELISA in medium collected from colonic organ culture as described previously (25).

**Cell culture.** As described previously (21), HCT116 cells were transfected for 72 h with a pEGFP plasmid with and without the MMP9 gene. Transfected clones were selected under an antibiotic (Geneticin, Gibco, Grand Island, NY), and fluorescent cells were isolated using flow cytometry (FACS), then used for the detection of IL-8. Stably transfected HCT116 cells were maintained in McCoy’s 5A medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 800 μg/ml Geneticin at 37°C in atmosphere containing 5% CO2.

**Statistical analysis.** The data are presented as means ± SE. Groups were compared by Student’s t-test. P values <0.05 were considered statistically significant.

**RESULTS**

Characterization of transgenic mice with intestinal epithelial cell-specific overexpression of MMP9 (Tg-villin-MMP9). To investigate the mechanism by which epithelial-derived MMP9 mediates inflammation, we generated transgenic mice with intestinal epithelial cell-specific MMP9 overexpression (Tg-villin-MMP9). Tg-villin-MMP9 mice were found to be healthy; body weight, breeding, and general appearance were normal. Tg-villin-MMP9 mice showed no evident phenotype upon observation of the intestine, including no change in colon length and colon weight. Tg-villin-MMP9 showed a significant (9-fold ± 1.23) increase in human MMP9 m-RNA levels compared with WT littermates (Fig. 1A). There was also a significant increase in MMP9 protein expression (5.7 ± 0.06) among Tg-villin-MMP9 mice compared with WT mice (Fig. 1B). Zymography data (not shown) indicated that Tg-villin-MMP9 mice did not have enzyme activity. Hematoxylin and eosin (H&E) staining for Swiss rolls of colon showed that both Tg-villin-MMP9 as well as WT mice had intact crypt architecture and muscularis mucosa layer (Fig. 1C), although an influx of neutrophils was observed among Tg-villin-MMP9 mice compared with WT mice (Fig. 1C). Our laboratory has previously (22) reported that MMP9 plays an important role in epithelial cell differentiation by mediating the preferential differentiation of progenitor cells to enterocytes. To examine the effect of constitutive expression of MMP9 in intestinal epithelial cells we performed Alcian blue-periodic acid Schiff (AB-PAS) staining as mentioned in MATERIALS AND METHODS to account for goblet cells. Figure 1D represents the AB-PAS staining of the colonic tissues of Tg-villin-MMP9 mice (right) vs. WT mice (left). Arrows indicate the purple mucin granules as well as blue-colored goblet cells and show that there is significant decrease in the number of goblet cells in Tg-villin-MMP9 mice compared with WT mice. The adjacent graph (Fig. 1E) shows the percentage of the number of goblet cells per crypt and indicates that Tg-villin-MMP9 mice had significantly lower number of goblet cells (53.87 ± 1.45%) compared with WT mice (72.87 ± 2.77%). Alteration in goblet cells may result in defective barrier function and mucin production. Hence we examined barrier function in Tg-villin-MMP9 and WT mice using a FITC-labeled dextran method, as described in MATERIALS AND METHODS. Figure 1F shows Tg-villin-MMP9 presence of 3.16 ± 0.82 mg FITC·μg protein⁻¹·h⁻¹, which was approximately threefold higher in FITC-dextran levels in WT mice (1.01 ± 0.16 mg FITC·μg protein⁻¹·h⁻¹), suggesting increased permeability or compromised barrier function in transgenic mice. We performed Muc-2 staining as illustrated in MATERIALS AND METHODS. The

Fig. 3. Intestinal epithelial cell-specific overexpression of MMP9 is associated with altered mRNA levels of proinflammatory cytokine Kc at the basal level. RNA was extracted from colonic mucosal stripping of Tg-villin-MMP9 mice and their WT littermates and mRNA levels were quantified using real-time PCR. A: Kc mRNA. B: IL-6 mRNA. C: TNF-α mRNA. D: IFN-γ (IFγ) mRNA. Each bar represents means ± SE, n = 5, *P < 0.05.
data indicate that Tg-villin-MMP9 mice showed significantly decreased mucin secretion as depicted by brown staining compared with WT mice (Fig. 1G). These results together imply that intestinal epithelial cell specific overexpression of MMP-9 is associated with defective barrier function as well as altered mucin secretion.

Intestinal epithelial cell-specific overexpression of MMP9 resulted in increased proliferation and apoptosis at the basal level. The next step was to identify the effect of constitutive expression of MMP9, on proliferation and apoptosis, which are the two key cellular processes. Proliferation is one of the major cellular processes, which gets altered during IBD and subsequently affects the cellular equilibrium. We performed Ki67 staining as illustrated in materials and methods to evaluate proliferation. The data indicate that Tg-villin-MMP9 mice showed increased proliferation as depicted by brown-colored nuclei compared with WT mice (Fig. 2A). The adjacent graph (Fig. 2B) shows the percentage of number of Ki67-positive nuclei per crypt and indicates that Tg-villin-MMP9 mice had significantly higher number of proliferating cells (31.98 ± 2.53%) compared with WT mice (19.84 ± 1.95%). TUNEL staining was performed to assess apoptosis in the colonic epithelium of Tg-villin-MMP9 mice. Figure 2C shows that there was an increase in apoptosis among Tg-villin-MMP9 mice compared with WT mice as indicated by green-colored fluorescent nuclei. Fig. 2C, middle shows the fluorescent blue DAPI nuclear staining and Fig. 2C, right shows the merged image of TUNEL and DAPI. Adjacent graph (Fig. 2D) shows

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**Figure 4.** Intestinal epithelial cell-specific overexpression of MMP9 mediates inflammation in DSS-induced colitis. As described in materials and methods, Tg-villin-MMP9 mice and their WT littermates were exposed to dextran sodium sulfate (DSS)-induced colitis. A: graphical presentation of change in body weight of Tg-villin-MMP9 mice and their WT littermates treated with DSS. B: bar graph presentation of the colonic length. C: bar graph presentation of clinical score based on the 3 parameters mentioned in materials and methods. D: survival curve. E: H&E staining (×10 magnification) of Swiss rolls of colon showing the extent of inflammation and crypt damage. F: bar graph presentation of histological score based on the 3 parameters mentioned in materials and methods. G: bar graph presentation of myeloperoxidase (MPO) activity. Values are representative of 3 experiments; each bar represents means ± SE (n = 6/group), *P < 0.05.
the percentage of number of apoptotic cells per crypt and indicates that Tg-villin-MMP9 mice had significantly higher number of apoptotic cells (4.8 ± 0.56%) compared with WT mice (0.33 ± 0.10%). These results highlight the fact that constitutive overexpression of MMP9 results in increased proliferation, which might be a secondary effect of mild inflammation, and to maintain the epithelial equilibrium it is also associated with compensatory increase in apoptosis.

These results together indicate the MMP9 overexpression in colonic epithelium is associated with a decrease in the differentiation of progenitor cells to goblet cells. Furthermore, overexpression of MMP9 in colonic epithelium does alter the two processes (proliferation and apoptosis) but overall maintains the equilibrium between the two.

**Intestinal epithelial cell-specific overexpression of MMP9 is associated with altered mRNA levels of proinflammatory cytokine Kc at the basal level.** Cytokines play a major role in the pathophysiology of IBD (9). The effect of constitutive expression of MMP9 in intestinal epithelial cells on proinflammatory cytokines IL-6, TNF-α, IFN-γ, and Kc was ascertained as one of the possible mechanisms by which MMP9 may mediate inflammation in IBD. mRNA levels of the above-mentioned proinflammatory cytokines were measured by quantitative PCR assays as mentioned in MATERIALS AND METHODS. There was a significant increase (3.70 ± 1.25) in mRNA levels of cytokine Kc in Tg-villin-MMP9 mice compared with WT mice (0.65 ± 0.23) (Fig. 3A). Figure 3, B–D, shows that there was almost no change in the mRNA levels of cytokines IL-6, TNF-α, and IFN-γ among Tg-villin-MMP9 mice compared with WT mice at the basal level. These results imply that overexpression of MMP9 in colonic epithelium does modulate the mRNA levels of proinflammatory cytokine Kc but not the mRNA levels of cytokines IL-6, TNF-α, and IFN-γ.

**Intestinal epithelial cell-specific overexpression of MMP9 mediates inflammation in DSS-induced colitis.** The pathogenesis of colitis in Tg-villin-MMP9 mice 3% DSS was examined by a DSS-induced colitis model as described in MATERIALS AND METHODS. Both Tg-villin-MMP9 and WT mice exposed to 3% DSS developed signs of colitis in 7 days and were euthanized. Mice were weighed daily and stool was examined for consistency and the presence of blood. Western blot of protein lysates prepared from the colonic tissue and H&E staining of the Swiss rolls of colon were performed as described in MATERIALS AND METHODS. Figure 4A represents the loss of body weight among Tg-villin-MMP9 mice, indicating that there was a significant decrease (91.8 ± 1.8%) compared with WT mice (99.21 ± 1.5%) during DSS-induced colitis. Severity of inflamed colon was reflected by shortened length and is represented in Fig. 4B. The data indicate that Tg-villin-MMP9 have significantly shorter colon length (5.8 ± 0.3 in cm) compared with WT mice (7.9 ± 0.6 in cm) in DSS-induced colitis. Figure 4C represents the clinical score, calculated on three parameters: loss in body weight, fecal consistency, and presence of blood in feces (as mentioned in MATERIALS AND METHODS). The graph in Fig. 4C shows that Tg-villin-MMP9 mice had significantly higher clinical scores (9.5 ± 0.5) compared with WT mice (6.5 ± 0.56) in DSS-induced colitis. In another set of experiments, we followed Tg-villin-MMP9 and WT mice (mice, n = 8 each group) for an additional 10 days after 7 days of DSS cycle to assess mortality. Mice were weighed every day and their mortality is represented in Fig. 4D, which shows the survival curve of Tg-villin-MMP9 and WT mice. At day 5, 13% of both
Tg-villin-MMP9 and WT mice given the DSS started rectal bleeding, reflecting the severity of inflammation. This was followed by a significant weight loss starting at day 7. At day 10, there was 21% mortality among Tg-villin-MMP9 group vs. 13% mortality among WT mice. Further extrapolation of DSS-induced colitis showed that at day 11 there was 21% mortality among Tg-villin-MMP9 group vs. 13% mortality among WT mice. At day 10, there was 57% mortality among Tg-villin-MMP9 mice vs. 38% mortality among WT mice. Overall, these data indicate higher mortality among Tg-villin-MMP9 mice compared with WT. Inflammation was also measured by MPO (an index of calculating neutrophil influx). Figure 4E represents the H&E staining of the Swiss rolls of colons and indicates that in DSS-induced colitis there was complete loss of crypt architecture and massive infiltration of neutrophils among Tg-villin-MMP9 mice compared with WT mice. Also, the ulcers were greater in number and involved larger surface area among Tg-villin-MMP9 mice compared with WT mice (Fig. 4F). Figure 4F shows significantly higher histological score (7.17 ± 0.48) among Tg-villin-MMP9 mice compared with WT mice (3.6 ± 0.81), both given DSS. Figure 4G indicates that during DSS-induced colitis Tg-villin-MMP9 mice had significantly higher (1.87 ± 0.68 MPO unit/mg of protein) MPO activity compared with WT higher (0.49 ± 0.14 MPO unit/mg of protein) mice, reflecting the severity of inflammation among Tg-villin-MMP9 mice.

We next measured mRNA level of proinflammatory cytokines Kc, IL-6, TNF-α, and IFN-γ. Figure 5, A–D, shows that DSS-induced colitis resulted in a significant increase in Kc, IL-6, TNF-α, and IFN-γ mRNA levels (41.55 ± 16.41, 30.58 ± 7.68, 17.54 ± 3.96, and 40.6 ± 12.58-fold increase, respectively) among Tg-villin-MMP9 mice compared with WT mice treated with water. There was also statistically significant increase in mRNA levels of cytokines Kc, IL-6, and IFN-γ among Tg-villin-MMP9 mice compared with WT mice both induced with DSS colitis (Fig. 5, A, B, and D, respectively), although the increase in mRNA levels of cytokine TNF-α (Fig. 5C) between the two groups induced with DSS colitis was not significant.
These results together indicate that epithelial-derived MMP9 mediates DSS-induced colitis and is associated with a substantial increase in the proinflammatory cytokines Kc and IFN-γ.

**Epithelial cell-specific overexpression of MMP9 mediates inflammation in S.T.-induced colitis.** S.T.-induced colitis was used as another model to assess the pathogenesis of colitis in Tg-villin-MMP9 mice and to support the results obtained with DSS-induced colitis model. This model induces clinical and histological features of mouse colitis predominantly involving the cecum (4, 23). The advantage of choosing this model is that it recapitulates some aspects of clinical and histological human infection as well as acute flares of IBD, where mucosal pathogen interaction is thought to mediate the pathogenicity of the disease. We used oral infection with S.T. after pretreatment of mice with streptomycin as described in MATERIALS AND METHODS. Both Tg-villin-MMP9 and WT mice exposed to S.T. were weighed daily and were euthanized after 48 h. Western blot of protein lysates prepared from the cecum tissue as well as H&E staining of the cecum tissue were performed as described in MATERIALS AND METHODS. Figure 6A represents the loss of body weight among Tg-villin-MMP9 mice indicating that there was a significant decrease (to 94.25 ± 4.6%) compared with WT mice, both induced with S.T. colitis. Mice were killed 24 h after the administration of S.T. and ceca were removed and photographed (Fig. 6B), which indicates that Tg-villin-MMP9 mice were more susceptible to the infection. The ceca of all the mice infected with S.T. appeared pale and shriveled to a small size and were filled with purulent exudates. The cecum was processed for histological characteristics and MPO activity as described in MATERIALS AND METHODS. Severity of inflammation was also analyzed histologically as represented in Fig. 6C, which indicates that there was increased neutrophil migration, crypt damage, and extensive ulcers among Tg-villin-MMP9 mice compared with WT mice. Figure 6D presents the histological score, which was calculated on the basis of three parameters (as mentioned above): loss of crypt architecture, neutrophil migration, and foci of ulcers. The data (Fig. 6D) indicate that Tg-villin-MMP9 mice had significantly higher histological score (4.8 ± 0.68) compared with WT mice (3.38 ± 0.87) in Salmonella-induced colitis. Severity of inflammation was also measured by MPO activity of cecum tissues and is shown in Fig. 6E, which indicates that Tg-villin-MMP9 mice had significantly higher (5.24 ± 0.86 MPO unit/mg of protein) MPO activity compared with WT mice (3.41 ± 0.35 MPO unit/mg of protein). In another set of experiments, we followed Tg-villin-MMP9 and WT mice (mice, n = 8 each group) for Salmonella infection (without streptomycin pretreatment) to assess mortality. Mice were weighed every day and their mortality is represented by survival curve in Fig. 6F. At day 6 14% of Tg-villin-MMP9 with S.T. infection died while only 13% WT mice died at day 8. There was 28% mortality among Tg-villin-MMP9 mice at day 10, which sharply dropped to 84% by day 11. On the other hand, WT mice showed 25% mortality at day 10, which dropped to 50% by day 12.

We also measured mRNA level of proinflammatory cytokines Kc, IL-6, TNF-α, and IFN-γ. Figure 7, A–D, shows that S.T.-induced colitis resulted in a significant increase in Kc, IL-6, TNF-α, and IFN-γ mRNA levels (418.27 ± 98.27, 2.99 ± 0.77, 24.62 ± 5.61, and 55.27 ± 6.2-fold increase, respectively) among Tg-villin-MMP9 mice compared with WT mice treated with water. There was also statistically significant increase in mRNA levels of cytokines Kc, IL-6, TNF-α, and IFN-γ among Tg-villin-MMP9 mice compared with WT mice both induced with DSS.
colitis (Fig. 7A, 7B, 7C, and 5D, respectively). Interestingly, there was massive increase in mRNA levels of cytokine Kc (Fig. 7A) between the two groups induced with S.T. colitis.

These results together imply and corroborate the DSS-induced colitis data that epithelial-derived MMP9 mediates acute colitis, which is associated with significant increase in the cytokine Kc.

**Epithelial cell-specific overexpression of MMP9 is associated with increased levels of cytokine Kc.** We next measured the expression of Kc (human homologue IL-8) by organ culture ELISA as described in MATERIALS AND METHODS. Figure 8A shows that there was significantly increased expression of Kc among Tg-villin-MMP9 mice (2.61 ± 0.18) compared with WT mice (1.28 ± 0.25) at the basal level. Similarly, Fig. 8B shows significantly increased expression of chemokine IL-8 among stably transfected HCT116 colonic epithelium cell line overexpressing MMP9 (3.3 ± 0.56) compared with vector (1.46 ± 0.14).

**DISCUSSION**

IBD is a complex and chronic condition resulting from dysregulated genetic and environmental factors. The pathophysiology of IBD is the combination of the individual role played by commensal microbiota, epithelial cells, and the immune system. In the United States specifically, an estimated 1 million individuals have IBD, with about 30,000 new cases reported each year (26). The incidence is evenly divided between UC and CD. MMPs being able to degrade ECM are involved in mediating inflammation as well as tissue repair in IBD (30). Furthermore, MMP9 is a well-known MMP actively involved in mediating inflammation in acute colitis, which has been well documented in literature although the precise role and the underlying mechanism by which it mediates acute colitis is still unknown.

Our laboratory has previously reported that MMP9 mediates inflammation in acute colitis by two different models, chemical as well as bacterial (12, 25). Our laboratory has also demonstrated through bone marrow chimera study that epithelial-derived MMP9 is responsible for mediating inflammation (12).

In the present study we have used genetically engineered mice (Tg-villin-MMP9 mice) that can constitutionally overexpress MMP9 specifically in epithelial cells to delineate the role/mechanism by which MMP9 mediates inflammation in acute colitis. We observed that intestinal epithelial cell-specific MMP9 overexpression is associated with decreased expression of goblet cells and defective barrier function but with increased expression of proliferation and apoptosis at the basal level. We also observed that there was a significant increase in the mRNA as well as protein levels of proinflammatory cytokine Kc (human homologue is IL-8). Both DSS (chemical)-induced and *Salmonella* (bacteria)-induced colitis models showed the worsening of acute colitis in Tg-villin-MMP9 mice compared with WT mice as evident by more weight loss and higher clinical score, histological score, and MPO values. Cytokine profile also suggested a significant increase in mRNA levels of proinflammatory cytokines IL-6, IFN-γ, and TNF-α, whereas a huge increase in the mRNA levels of proinflammatory cytokine Kc was observed among Tg-villin-MMP9 mice compared with WT mice. These results together signify that epithelial-derived MMP9 may mediate inflammation in acute colitis through impaired barrier function. Further increased levels of proinflammatory cytokine Kc might be due to dysregulation of barrier function or may be due to proteolytic action of MMP9 leading to leakage of Kc (mouse homologue of IL-8).

Mucins are the important component of the GI tract and serve as the first line of defense to infection. Genetic defects in intestinal goblet cells can lead to spontaneous inflammation (33). In addition to mucosal barrier defense, the GI tract also secretes immunologically active substances that modulate mucosal inflammatory and immune responses (41).

Proinflammatory cytokines are immunomodulating agents. It has been well documented in literature that cytokines are involved in the dysregulated inflammatory response in IBD, although the exact mechanism is still unknown. Different studies have reported that IL-8 (mouse homologue is Kc), which is a potent cytokine for recruitment and activation of neutrophils and plays an active role in the induction and promotion of IBD (16, 32, 34, 49, 52, 53), although none of these studies could delineate the exact mechanism by which IL-8 mediates or propagates inflammation in IBD. Furthermore, Singer and Sansonetti (46) demonstrated that intraluminal addition of recombinant IL-8 was directly associated with the development of acute invasive *Shigella*-induced colitis. Berin et al. (8) reported that enterohemorrhagic *Escherichia coli* O157:O7 (EHEC) infection of human intestinal epithelial cells activates MAP kinase and NF-κB signaling pathways, thereby activating the expression and production of IL-8 in human intestinal epithelial cells (8, 54, 56). Ju et al. (27) have shown that in gastric cancer IL-8 mediates cell migration and invasion.

Although IL-8 can be expressed by CD14+ macrophages, neutrophils, endothelial cells, fibroblasts, T lymphocytes, and
epithelial cells, it has been reported that intestinal epithelial cells isolated from mucosa of IBD patients specifically expressed IL-8 (2, 37), suggesting epithelial cells as one of the central player in the complex regulatory network for proinflammatory cytokine IL-8.

A direct connection between MMP9 release and IL-8 activation has been observed in different organ systems. A direct interaction between MMP9 and IL-8 has been reported in hematopoietic stem cell trafficking in liver injury (17, 20), in retinoic acid syndrome (45), and in leucocytes (51). Beklen et al. (7) showed that there was an upregulation of both MMP9 and IL-8 in periodontitis. Many studies related with airway epithelium have shown a direct link between the IL-8 secretion and MMP9 expression (15, 31, 55). Yoon et al. (55) have shown that MMP9 protects from ozone induced airway injury may be via posttranscriptional effects on IL-8. In chronic obstructive pulmonary disease and leucocytes, it has been reported that IL-8, being a CXC chemokine, interacts with the two receptors: CXCR1 and CXCR2 of the CXCR family (3, 13, 51).

Williams et al. (53) have reported the presence of these two receptors in normal GI tract and also about their modification in inflamed gut tissue. They observed that CXCR1 are strongly upregulated in the mucosal epithelium, specifically and commonly on morphologically damaged surface epithelium (53), whereas the role and distribution of CXCR2 in GI tract has not been well explored. Recently, Shea-Donohue et al. (44) have shown the protective role of the CXCR2 ligand CXCL1 in DSS-induced colitis in murine model, which, however, contradicts the findings reported by Buanne et al. (11). Therefore, the precise role and distribution of these receptors in GI tract in acute colitis model is not well established.

Our study for the first time have demonstrated that colonic epithelial-derived MMP9 acts as a regulatory molecule for IL-8 expression and thereby mediates inflammation in acute colitis. Our hypothesis has also been supported by the study done by Van Den Steen et al. (50, 51), where they have shown that neutrophil-derived MMP9 cleaves IL-8 and potentiates its activity. This reveals the presence of an important positive feedback loop between MMP9 and IL-8, suggesting that MMP9 not only acts as an effector molecule but also acts as a regulatory molecule.

Our study demonstrates through a transgenic mice model that intestinal epithelial cell-specific overexpression of MMP9 in colon is directly correlated with defective barrier function, altered mucin secretion, and increased IL-8 mRNA and protein expression at the basal level. We also showed that epithelial cell-specific overexpression of MMP9 worsens acute colitis as evident by chemical- and bacterial-induced colitis models. Interestingly, in both these models cytokine profile data also indicated that there was a huge increase in the mRNA levels of proinflammatory cytokine Kc (human homologue is IL-8) compared with others like IL-6, TNF-α, and IFN-γ. Together, the data show that MMP9 in colonic epithelium causes spontaneous inflammation associated with altered Muc2 level and an increase in the levels of proinflammatory chemokine Kc. Increased levels of Kc can be explained as being either due to spontaneous inflammation and/or due to its cleavage by pro tease MMP-9.

REFERENCES


