Increased activity and expression of matrix metalloproteinase-9 in a rat model of distal colitis

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Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent endopeptidases that are capable of degrading most components of the extracellular matrix (ECM). These enzymes are involved in the remodelling and degradation of ECM during physiological and pathological conditions. Most MMPs are secreted aszymogens that require proteolytic activation of latent protein. In addition, the activity of MMPs is strictly regulated by specific tissue inhibitors of MMP (TIMPs) (3, 15, 24, 25). The MMPs have been classified into collagengases, gelatinases, and stromelysins based on the substrate specificity of individual MMPs. Collagenases have the ability to digest interstitial collagens of type I, II, and III, and they are mainly synthesized by connective tissue cells, macrophages, and polymorphonuclear leucocytes. Gelatinases may degrade type IV collagen and gelatine. Two gelatinases have been identified: the 72-kDa proenzyme pro-MMP-2 and the 92-kDa proenzyme pro-MMP-9. The stromelysins have a much broader substrate specificity and degrade a wide range of ECM components, including proteoglycans, type IV collagen, fibronectin, and laminin (3).

In some inflammatory reactions such as rheumatoid arthritis (10) and periodontal disease (14), increased expression of MMPs has been observed. This increase can result in accelerated breakdown of the ECM and may contribute to the pathogenesis of these disease states. MMPs can be released by several cells in response to proinflammatory cytokines such as interleukin-1 and tumor necrosis factor-α. In addition to inflammation, MMPs have been also implicated in tumor growth and metastasis (15). For these reasons, synthetic MMP inhibitors such as marimastat and batimatast, are currently being tested in patients with neoplasm and other conditions in which MMPs have been pathophysiological implicated.

Ulcerative colitis is a chronic inflammatory bowel disease whose etiology still remains unknown. Features of ulcerative colitis include diffuse mucosal inflammation of the distal colon and rectum. Increased proteolysis of mucosal tissue in these patients has been suggested by a previous study (13) as manifested by reduced deposition of types I and III collagen contrasted with an elevated level of RNA transcripts for these proteins. MMP-9 may be a key enzyme responsible for the accelerated breakdown of ECM in ulcerative colitis, because it was demonstrated that MMP-9 is abundantly expressed in patients with ulcerative colitis compared with controls (2).
The objective of our study was to investigate the activity and expression of MMP-2 and MMP-9 in a rat model of colitis induced by dextran sulphate sodium (DSS) and to test the effect of a synthetic MMP inhibitor, CGS-27023-A, on tissue damage. The DSS rat model exhibits clinical and morphological features resembling human ulcerative colitis, including diarrhea and rectal bleeding, diffuse lesions circumscribed to the mucosa, and predominance of distal involvement of the large intestine.

METHODS

Animals

Male Sprague-Dawley rats weighing 180–200 g were purchased from Centre d’Elevage R. Janvier (Le Genest, France). The animals were maintained in a restricted-access room with controlled temperature (23°C) and 12:12-h light-dark cycle and were housed in rack-mounted cages with a maximum of five rats per cage. Standard rodent chow pellets (Biocenter, Barcelona, Spain) and tap water were provided ad libitum. The local research committee (Comissio de Recerca, Hospital General Vall d’Hebron) approved the study.

Induction of DSS Colitis

Distal colitis was induced by oral DSS (molecular wt 40,000; ICN Biomedicals, Aurora, OH) at 5% (wt/vol) in tap water ad libitum for 5 days in the colitis group (n = 8) (16), whereas the control group (n = 8) only received plain water. Body weight was routinely obtained every second day. Sets of four rats were euthanized on days 3 and 5 after induction of colitis. With the use of sterile equipment, a midlaparotomy was performed, the colon was removed, opened longitudinally, rinsed with sterile saline, and divided into two parts by a longitudinal section. One specimen was homogenized and stored at −20°C for MMPs and MPO assay. The second specimen was used for microscopic assessment of mucosal lesions.

Effects of MMP Inhibition in Rats with DSS Colitis

Mucosal damage. Twenty rats were included in this protocol. Distal colitis was induced in all rats by DSS at 5% in drinking water for 5 days. The control group consisted of 10 rats that received oral gavage every day after induction of colitis. The test group consisted of 10 rats that received CGS-27023-A by oral gavage at a dose of 20 mg·kg⁻¹·day⁻¹ from day 0 to 5. The compound CGS-27023-A [N-hydroxy-2-(R)-4-methylsulfonyl)-3-picolyl)-amino-3 methylbutaneamide hydrochloride monohydrate] is a potent, orally available, non-peptidic synthetic inhibitor of MMPs activity (12). A sample of CGS-27023-A was supplied by Lacer (Barcelona, Spain) for the purpose of this study. Body weight was routinely obtained every second day. Rats were euthanized by cervical dislocation on day 5 after induction of colitis, and the colon was removed, opened longitudinally, rinsed with sterile saline, and divided into two parts by a longitudinal section. One specimen was homogenized for MPO assay, and the second was processed for histological assessment of mucosal lesions.

Mucosal repair. Twenty rats received DSS at 5% in drinking water from day 0 to 5. The control group consisted of 10 rats dosed orally with vehicle from day 0 to 14. The test group consisted of 10 rats that received CGS-27023-A by oral gavage at a dose of 20 mg·kg⁻¹·day⁻¹ from day 0 to 14. Rats were euthanized by cervical dislocation on day 14. A mid-laparotomy was performed, and the colon was removed, opened longitudinally, rinsed with sterile saline, and fixed in formalin for microscopic assessment.

Analytical Methods

Zymography. The activity of MMP-2 and MMP-9 was measured as previously described (5, 19) in homogenates of colonic tissue. Briefly, colonic samples were homogenized and mixed with electrophoresis loading buffer (10:1; vol/vol). Thereafter, samples were subjected to electrophoresis on 7.5% SDS-PAGE copolymerized with gelatine (2 mg/ml). After electrophoresis, gels were washed with 2.5% Triton X-100 for 1 h (3 times, 20 min each) and incubated for 48 h in enzyme assay buffer (25 mM Tris, pH 7.5, 5 mM CaCl₂, 0.9% NaCl, 0.05% Na₂N) for the development of enzyme activity bands. After incubation, the gels were stained with 0.05% Coomassie brilliant blue G-250 in a mixture of methanol-acetic acid-water (2:5:1.6:5) and destained in 4% methanol with 8% acetic acid. The gelatinolytic activities were detected as transparent bands against the background of Coomassie brilliant blue-stained gelatine. MMPs were identified by their molecular weight compared with standards and the susceptibility to inhibition with MMP inhibitor phenantroline (5, 19). To measure the activities of the detected enzymes, zymograms were read using a ScanJet 3c scanner (Hewlett-Packard, Boise, ID). The intensities of the separate bands were analyzed using SigmaGel measurement software (Jandel, San Rafael, CA). The activity of MMPs was normalized to the protein content in the sample.

Western blot. Western blot analysis of MMP-9 was performed as previously described (5). Briefly, samples were subjected to 7.5% SDS-PAGE. After electrophoresis, they were electroblottedted onto polyvinylidene fluoride membranes (Schleicher and Schuell, Keene, NH) and probed with polyclonal antibodies against MMP-9 (5). Bands corresponding to pro-MMP-9 were identified with an enhanced chemiluminescence Western blotting detection kit (Amersham Life Science, Buckinghamshire, UK), read, and quantified using a ScanJet scanner and SigmaGel software. To account for the interblot variations in MMPs immunoreactivity, internal standard (conditioned medium of HT-1080 human fibrosarcoma cells that contains high amounts of pro-MMP-2, MMP-2, pro-MMP-9, and MMP-9) was used, and the results were standardized by comparison with the standard (19).

MPO assay. For the assay of MPO activity, the colonic specimen was homogenized in 2 ml phosphate-buffered saline, using a Tissue Tearor (model 985–370; Biospec, Racine, WI), and centrifuged (20). The pellets were again homogenized in an equivalent volume of phosphate buffer (50 mM, pH 6) containing 0.5% hexadeceyltrimethylammonium bromide (Sigma) and 5 mM EDTA, sonicated three times for 30 s each time (Labasonic 2000; Braun), and centrifuged. Supernatants were used for determination of tissue MPO activity by a kinetic method. One unit of enzyme activity is defined as the amount of MPO that degrades 1 mmol of peroxide per minute at 25°C.

Histological Assessment of Colonic Lesions

Colonic specimens were fixed in formalin and coded for blind microscopic assessment of mucosal lesions. Samples were embedded in paraffin using a “Swiss roll” technique, and longitudinal sections from cecum to rectum were prepared and stained with haematoxylin and eosin. This procedure allows the examination of the entire specimen from cecum to rectum in every section. A pathologist who was unaware of the treatment measured the extent of mucosal damage.
surface involved by crypt lesions as a percentage of the total colonic surface. For each specimen, a crypt damage score was obtained by summation of the products of each grade of crypt damage times the extent of mucosal surface involved by this grade of damage (see Table 1). In addition, the specimens were graded 0 to 3 for inflammation (acute/chronic), regenerative changes (hyperplastic epithelium) and crypt distortion (distorted epithelium) using the criteria described by Cooper et al. (4). Acute inflammation scores evaluate the presence of polymorphonuclear neutrophils in the infiltrate, whereas chronic inflammation scores evaluate the presence of mononuclear cells.

**Statistical Methods**

Results are presented as means ± SE. Statistical difference between control and test group was determined by Student’s t-test. A two-tailed P value <0.05 was considered as statistically significant. The correlation between MMP activity and injury was examined by Spearman’s coefficient of rank correlation.

**RESULTS**

**DSS Colitis**

Exposure to 5% DSS in drinking water for 5 days induced diarrhea and rectal bleeding in all rats. In addition, oral administration of DSS resulted in a significant increase in MPO activity in the colonic tissue (2.1 ± 0.5 U/g) compared with normal control rats (0.9 ± 0.1 U/g). Histological examination of colonic sections revealed that oral administration of DSS at this concentration resulted in severe injury of the colonic epithelium and loss of normal crypt architecture, with some areas of erosion. Mixed infiltrate of neutrophils and mononuclear cells was observed in the lamina propria and submucosa. The muscularis propria was not involved. These changes were diffuse but predominated at distal parts of the colon. Histological examination revealed no changes in colons from normal control rats.

**MMP Activity in DSS Colitis**

Figure 1A shows zymograms of colonic homogenates from control and colitic rats treated with DSS for 5 days. In control animals, pro-MMP-2, but not pro-MMP-9, was a dominant gelatinase. In fact, all control rats showed negligible activity of pro-MMP-9, with a median of 0.25 ± 0.25 density units per 5 μg of protein. Induction of colitis resulted in the appearance of pro-MMP-9 in addition to pro-MMP-2 activity (Fig. 1A). The densitometric analysis (Fig. 1B) showed a

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**Table 1. Microscopic assessment of histological changes in DSS colitis**

<table>
<thead>
<tr>
<th>Grade of crypt damage</th>
<th>Score</th>
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<tbody>
<tr>
<td>Intact crypt</td>
<td>0</td>
</tr>
<tr>
<td>Grade 1: loss of basal third</td>
<td>1</td>
</tr>
<tr>
<td>Grade 2: loss of two thirds</td>
<td>2</td>
</tr>
<tr>
<td>Grade 3: loss of entire crypt</td>
<td>3</td>
</tr>
<tr>
<td>Grade 4: erosion</td>
<td>4</td>
</tr>
</tbody>
</table>

Extent of each grade of crypt damage as % total mucosal surface: 0–100

Crypt damage score = Σ(A × B)*

Grade of inflammation, epithelial regeneration or crypt distortion:

- Absent: 0
- Mild: 1
- Moderate: 2
- Severe: 3

Crypt damage, acute and chronic inflammation, epithelial regeneration, and crypt distortion are scored separately. DSS, dextran sulphate sodium. *A, grade of crypt damage; B, extent of each grade of crypt damage as % total mucosal surface.
significant upregulation of pro-MMP-9 ($P < 0.01, n = 4$) but not pro-MMP-2 ($P > 0.05, n = 4$) activity in colitic rats on day 5 when compared with control rats.

At earlier times in disease, 3 days after the onset of DSS, there were no significant differences in pro-MMP-9 activity between control rats ($0.36 \pm 0.13$ units/5 $\mu$g protein, $n = 4$) and rats treated with DSS ($5.60 \pm 2.43$, $n = 4$; $P = 0.119$).

**MMP Expression in DSS Colitis**

Figure 2A shows Western blot analysis of colonic homogenates from controls and rats with colitis induced by DSS on day 5 after exposure to DSS. Pro-MMP-9 was found in colonic tissue homogenates from both control and colitic rats, as shown by the immunoreactive band migrating at $\sim 92$ kDa. The densitometric analysis demonstrated a significant ($P < 0.01, n = 4$) upregulation of pro-MMP-9 expression in rats treated with DSS for 5 days when compared with controls.

Figure 3 shows the relationship between the pro-MMP-9 activity measured by zymography and the crypt damage scores in rats with colitis induced by DSS. A close correspondence was observed between pro-MMP-9 activity and histological assessment of injury ($r = 0.9299; P < 0.001$).

**MMP Inhibition in DSS Colitis**

All DSS-treated rats developed diarrhea and rectal bleeding. In the first study, one rat from the vehicle control group and another one from the CGS-27023-A-treated group died during the follow-up, so that by the end of the study there were nine surviving rats in both groups. On day 5 after induction of colitis, rats treated with CGS-27023-A had body weight ($220 \pm 12$ g) similar to control rats ($205 \pm 13$ g).

The effect of CGS-27023-A on acute lesions induced by DSS (day 5) is shown in Fig. 4. Rats that received the synthetic MMP inhibitor showed significantly lower crypt damage scores than controls. On the other hand, there were no significant differences in acute (controls: $0.7 \pm 0.14$; CGS-27023-A: $0.6 \pm 0.17$) and chronic (controls: $0.7 \pm 0.14$; CGS-27023-A: $0.7 \pm 0.16$) inflammation scores. Epithelial regeneration and crypt distortion were almost absent at this stage, and no differences were found between controls and test rats.

![Fig. 2. Western blot detection of pro-MMP-9 protein in homogenates of colonic tissues. A: analysis in controls and rats with colitis induced by DSS. Western blot analysis showed marked expression of pro-MMP-9 in the homogenate of colitic samples. B: quantitative data (*$P < 0.05$ vs. control).](image)

![Fig. 3. Significant relationship between the activity of pro-MMP-9 and crypt damage scores in rats with colitis.](image)
Tissue MPO activity was similar in CGS-27023-A-treated (1.9 ± 0.3 U/g) and control (2.2 ± 0.5 U/g) rats. In the mucosal repair study, all rats survived during the 14-day follow-up period. Rats treated with CGS-27023-A showed significantly lower scores of crypt damage, acute inflammation, and chronic inflammation (Table 2). There were no differences in epithelial regeneration and crypt distortion scores.

**DISCUSSION**

Several lines of evidence suggest that MMPs are involved in the process of tissue destruction and remodelling associated with inflammatory conditions (10, 14). The aims of the current study were to investigate the activity and expression of MMPs in a rat model of colonic mucosal inflammation induced by DSS and to test the effect of a synthetic MMP inhibitor (CGS-27023-A) on severity of mucosal damage. In a rat model of DSS-induced colitis, our studies demonstrated upregulation of MMP-9 activity.

The presence of gelatinases (MMP-2 and MMP-9) was examined in colonic wall tissue from colitic and normal rats. Activated MMPs are rarely detected, on account of their fast degradation, and, therefore, changes in expression and activity of proforms were investigated. Zymographic analysis demonstrated no significant difference in pro-MMP-2 activity between normal rats and rats with colitis. In contrast, DSS-induced colitis resulted in substantial increases in pro-MMP-9 activity. Western blot studies confirmed the increased expression of pro-MMP-9 protein in colitic mucosa. In contrast, despite the presence of measurable pro-MMP-9 immunoreactive protein in normal colonic tissue, the activity of pro-MMP-9 was extremely low, suggesting that an endogenous inhibitor of MMP-9 prevents this enzyme from having a collagenolytic activity in a normal setting. These results concur with a previous report of increased MMP-9 and unchanged MMP-2 protein by immunohistochemical techniques in patients with inflammatory bowel disease (IBD) (1).

Several recent publications indicate that elevated levels of various MMPs can be found in inflamed colonic tissue from patients with IBD. Baugh et al. (2) found increased MMP-9 activity in patients with ulcerative colitis, both in involved and uninvolved areas. Von Lampe et al. (23) also reported a marked overexpression of MMP-1 and MMP-3 as assessed by mRNA levels in inflamed mucosal samples from patients with ulcerative colitis compared with controls. These results are also supported by recently published data from Heuschkel et al. (8), that demonstrated an over-expression of MMP-3 at inflamed sites, whereas TIMP-1 remained unaltered. In addition, Stallmach et al. (21) also support the hypothesis that MMPs are implicated in mucosal destruction associated with intestinal inflammation, since they found an increased concentration of MMP-1 and MMP-2 by sandwich enzyme linked immunosorbent assay in tissue from patients with active ulcerative colitis and patients with pouchitis.

To our knowledge, only one previous study (22) has reported MMP activity by zymography in an experimental model of colitis. In that model, colitis was induced in immunodeficient mice by transfer of CD4+ lymphocytes. Transmural inflammation was associated with serine proteinase and gelatinases (MMP-2 and MMP-9) activities in overlying epithelium. Zymographic analysis demonstrated a very significant increase of MMP-9 activity in colitic tissue specimens. Our results also show that colitis induced by DSS upregulates MMP-9 enzyme protein and its activity. Therefore, MMP-9 seems to be a major enzyme expressed in inflamed colon tissues. Our data are also consistent with human data reported recently by Baugh et al. (2), who showed that MMP-9 measured by zymography was the most abundant protease expressed in patients with ulcerative colitis compared with levels of normal controls. However, in our study, MMP-3 was not measured, and this enzyme is also likely to be involved in intestinal mucosal inflammation, as suggested by the human studies.

In our study, a close relationship was found between pro-MMP-9 activity and extent of injury in inflamed colonic tissue. This finding may suggest that increased expression and activity of MMP-9 is part of the mechanism of tissue injury in the DSS model of colitis. Direct evidence of the destructive effect of proteolysis

**Table 2. Histological scores on day 14 after induction of colitis by DSS**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CGS-27023-A</th>
</tr>
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<tbody>
<tr>
<td>Crypt damage</td>
<td>13.5 ± 2.04</td>
<td>5.9 ± 2.36*</td>
</tr>
<tr>
<td>Acute inflammation</td>
<td>1.70 ± 0.30</td>
<td>0.59 ± 0.20*</td>
</tr>
<tr>
<td>Chronic inflammation</td>
<td>1.15 ± 0.10</td>
<td>0.50 ± 0.16*</td>
</tr>
<tr>
<td>Epithelial regeneration</td>
<td>0.92 ± 0.10</td>
<td>1.10 ± 0.17</td>
</tr>
<tr>
<td>Crypt distortion</td>
<td>0.50 ± 0.16</td>
<td>0.55 ± 0.18</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. control.
by MMPs on mucosal organization has been gathered in a human fetal gut model (17), in which organ culture supernatants of explants stimulated with pokeweed mitogen showed a threefold increase in the concentration of MMP-1 and a 10-fold increase of MMP-3. In this model, nanomolar amounts of recombinant MMP-3 added directly to explants produced rapid severe tissue injury and pokeweed mitogen-induced mucosal damage was inhibited by a synthetic MMP inhibitor.

The important role of the MMPs in situations such as tumor progression and metastasis has prompted aggressive development of therapeutic agents that block enzyme activity in MMP-associated pathophysiological processes. We evaluated the effect of a broad-spectrum MMP inhibitor, CGS-27023-A (12), on tissue damage in our model of DSS-induced distal colitis. CGS-27023-A is a synthetic MMP inhibitor that has been tested successfully in several pathological conditions, such as tumor growth (7, 9, 11), progression of arteriosclerosis and aneurysm (18), and degradation of articular cartilage (6). Our study shows that daily oral administration of CGS-27023-A at doses of 20 mg·kg⁻¹·day⁻¹ substantially reduced colonic mucosal damage induced by exposure to DSS. Treated rats showed significantly lower crypt damage scores than controls. At early stages, CGS-27023-A did not affect tissue levels of MPO, suggesting that it did not affect recruitment of MPO-expressing inflammatory cells such as leukocytes into inflamed tissue. In the DSS model of colitis, inflammation is thought to be a secondary event that follows epithelial and crypt damage (4). We speculate that crypt damage occurring during the initial stage of DSS colitis may be due to an imbalance between degradative and reparative processes of ECM. Indeed, although several factors can influence such balance in favor of ECM degradation, the widespread activation of MMPs is likely to be a significant factor and the final step in the cascade of events resulting in tissue injury.

In the second study, CGS-27023-A was given for 14 days to investigate its effect on the mucosal repair process. Again, rats treated with the MMP inhibitor showed lower crypt damage scores. In addition, inflammation was lower in rats treated with the inhibitor, suggesting that epithelial protection also accounted for a mitigated inflammatory response. Interestingly, histological evidence of epithelial regeneration and crypt distortion was similar in both controls and rats treated with the MMP inhibitor. These findings suggest that the impact of MMP inhibition by CGS-27023-A on mucosal repair, if any, was not detectable by changes in tissue morphology.

In conclusion, our results suggest that increased expression and activity of MMPs may contribute to tissue injury in experimental ulcerative colitis induced by DSS.

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