

# Matrix metalloproteinase-9 modulates intestinal injury in rats with transmural colitis

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**Abstract:** Proteolysis and degradation of extracellular matrix by metalloproteinases (MMPs) may contribute to intestinal injury in inflammatory bowel disease. In the present study, we investigated the pathogenic role of gelatinases (MMP-9 and MMP-2) on transmural colonic injury in a rat model of chronic colitis, which was induced by intracolonic instillation of trinitrobenzene sulfonic acid (TNBS). The activity and expression of MMP-2 and MMP-9 were measured in colonic tissue and peripheral neutrophils by fluorescence, zymography, Western blot, or immunohistochemistry at different time-points. Furthermore, myeloperoxidase content in colonic homogenates was analyzed to evaluate inflammation. Finally, morphological changes were assessed following early or delayed administration of CGS-27023-A, a synthetic inhibitor of MMPs. We found that the induction of colitis led to a significant up-regulation in tissue gelatinase concentration, whereas no changes in collagenase activity were observed. In addition, up-regulation of pro-MMP-9, but not of pro-MMP-2, was found on Days 7 and 10 following the induction of colitis. Furthermore, transmural MMP-9 was detected by immunofluorescent staining in the inflamed tissue. Consistent with tissue samples, neutrophils from colitic rats showed a significantly increased activity of pro-MMP-9. Finally, early but not delayed treatment with CGS-27023-A attenuated colonic mucosal injury in rats with TNBS-induced colitis. In conclusion, up-regulation of MMP-9 in peripheral and colonic neutrophils modulates transmural colonic injury in rats with TNBS-induced colitis. *J. Leukoc. Biol.* 79: 954–962; 2006.

**Key Words:** inflammatory bowel disease · experimental colitis · trinitrobenzenesulfonic acid · myeloperoxidase

## INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) whose etiology still remains unknown. In the last decade, several experimental models of colitis have been developed in

an attempt to understand different pathophysiological mechanisms implicated in colonic inflammation. The trinitrobenzene sulfonic acid (TNBS) model of colitis is characterized by promoting chronic colitis with transmural inflammation and serosal involvement. In this experimental model, acute lesions consist of extensive necrosis of the mucosa, severe submucosal edema, and acute neutrophil infiltrate. Chronic changes may be observed from 1 to 3 weeks following TNBS administration [1, 2]. Lesions are segmental and well-circumscribed, consisting of mucosal ulcerations with granulation tissue at the base and mixed transmural infiltration by neutrophils, lymphocytes, and macrophages. Small granulomas are often observed in the submucosa and serosa. In severe TNBS colitis, transmural fibrosis causes stricture of the lumen, and uninvolved areas do not show mucosal inflammation.

Matrix metalloproteinases (MMPs) comprise an ever-growing family of zinc and calcium-dependent endopeptidases, which are involved in the remodeling and degradation of extracellular matrix (ECM) during physiological and pathological conditions, such as tumor growth, metastasis, and some inflammatory reactions [3–8]. The MMPs have been classified into collagenases, gelatinases, and stromelysins based on the substrate specificity. Collagenases have the ability to digest interstitial collagens of types I, II, and III. Two gelatinases, which degrade type IV collagen and gelatine, have been identified: the 72-kDa proenzyme pro-MMP-2 and the 92-kDa proenzyme pro-MMP-9. The stromelysins have much broader substrate specificity and degrade a wide range of ECM components [3–5]. Four endogenous tissue inhibitors of MMPs (TIMPs) strictly regulate the activity of these enzymes [3–5].

Recent studies have demonstrated an increased activity and expression of MMPs on colonic tissues in patients with CD compared with controls [9–12]. This increase might result in accelerated breakdown of the ECM and contribute to the pathogenesis of this disease state. In fact, increased proteolysis of mucosal tissue in IBD patients has been suggested by a previous study [13], as manifested by reduced deposition of

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types I and III collagen contrasting with an elevated level of RNA transcripts for these proteins.

The objective of the present study was to assess the pathogenic role of gelatinases (MMP-2 and MMP-9) in a rat model of transmural colitis.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats weighing 220–250 g were purchased from Centre d'Élevage R. Janvier (Le Genest, France). The animals were maintained in a restricted-access room with controlled temperature (23°C) and light-dark cycle (12 h:12 h) 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. Experimental protocols were approved by the Animal Care and Use Committee of Hospital Universitario de Canarias.

### Experimental colitis and procedures

Forty rats were included in this study. Transmural colitis was induced in five groups of seven rats each, whereas the control group comprised five rats, which received 1 ml saline enema. On days 3, 7, 10, 14, and 21 after colonic instillation, rats were killed by cervical dislocation. Using sterile equipment, a mid-laparotomy was performed, and the distal colon was removed, opened longitudinally, rinsed with sterile saline, and divided into two parts by a longitudinal section. One specimen was homogenized for MMPs and myeloperoxidase (MPO) assay. Briefly, colonic tissue (3–5 mg) was homogenized in 50 mmol/L Tris HCl (pH=7.6), 150 mmol/L NaCl (diluted 20-fold, vol/weight), and proteolytic enzyme inhibitors (Complete, Roche, Nutley, NJ). Samples were sonicated three times for 10 s each (UP100H, Dr. Hielscher, Germany) at 1-min intervals. After 10 min on ice, protein extracts were centrifuged for 10 min at 13,000 g at 4°C, the supernatants were preserved, and protein concentrations were determined by the bicinchoninic acid method (Sigma Chemical Co., St. Louis, MO). Samples were stored at –80°C until assayed. The second specimen was used for assessment of colonic damage and immunohistochemistry.

### Activity and expression of MMPs

Total gelatinase and collagenase activity was measured in homogenates of colonic tissue by fluorometry, as described previously [14]. For this purpose, the EnzChek gelatinase assay kit (Molecular Probes, Eugene, OR), a fibril degradation assay, which used self-quenched, fluorescein-conjugated gelatin and type I collagen, was used. When these substrates were digested, highly fluorescent peptides were released and detected by fluorometry. The assay was performed following the manufacturer's recommendation, and the fluorescent intensity was measured in duplicates with a spectrofluorometer Genios (Tecan, Austria). Data were expressed as MMP activity in mU collagenase per mg total protein. The MMP inhibitor, 1, 10-phenantroline at 0.1 mmol/L, was added to the development buffer to test the specificity of the fluorometric assay.

The activity of MMP-2 and MMP-9 was measured in homogenates of colonic tissue and in peripheral neutrophils by zymography, as described previously [15, 16]. For the isolation of peripheral neutrophils, a blood sample was collected from the abdominal aorta. Purified neutrophils were obtained by isolation on Histopaque 1077 (Sigma Ltd., UK), and erythrocyte contaminants were removed by hypotonic lysis, as described previously [17]. The count of neutrophils was determined by using a Neubauer hemocytometer, and cell viability was assessed by trypan blue exclusion. Typically, the neutrophil preparations were >98% pure and >94% viable. The isolated neutrophils were washed and resuspended in Hanks' balanced saline solution with calcium and magnesium in a concentration of  $5 \times 10^6$  cell/mL and incubated for 12 h at 37°C. MMP release was determined in the supernatant by zymography and immunoblots for MMP-9 and MMP-2.

Zymography was performed, subjecting samples to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and copolymerized gelatin (0.2%, Sigma Chemical Co.) was incorporated as a substrate for gelatinolytic proteases. After electrophoresis, the gels were washed with 2%

Triton X-100 (2 times, 20 min each) and then incubated in development buffer (50 mmol/L Tris HCl, 200 mmol/L NaCl, 10 mmol/L CaCl<sub>2</sub>, and 1 μmol/L ZnCl<sub>2</sub>, pH=7.5) at 37°C overnight. Serine protease inhibitor (2 μg/mL aprotinin) and MMP inhibitors (10 mmol/L EDTA and 0.1 mmol/L o-phenantroline) were added to the development buffer to assess whether the observed gelatinolytic bands were caused by serine protease activity or MMP activity. Internal standard, recombinant human MMP-9 and MMP-2 (Oncogene Research, Nottingham, UK) and conditioned medium of HT-1080 human fibrosarcoma cells (containing high amounts of pro-MMP-2, MMP-2, pro-MMP-9, and MMP-9) were run as controls on the gels. After 18 h development, gels were fixed and stained in 40% methanol, 10% acetic acid, and 0.1% (wt/vol) Coomassie Blue R-250 (Sigma Chemical Co.) for 1 h and then destained. Relative molecular weights of clear bands were analyzed in comparison with molecular weight standards (Dual Color, Bio-Rad, Hercules, CA), purified human MMP-2 and MMP-9, and conditioned medium of HT-1080 human fibrosarcoma cells, using a calibrated densitometer (GS-800, Bio-Rad) and Quantity One Quantitation analysis software (Version 4, Bio-Rad). Zymographic activity of each band was measured in terms of optical density units (ODu) of trace quantity/mm.

As MMP-9 activity was enhanced markedly in homogenates of colonic tissue and blood samples of rats with TNBS-induced colitis, we tested the expression of this gelatinase in colonic homogenates by Western blot analysis [16]. For this purpose, samples were denatured in reducing treatment buffer and loaded (50 μg/lane) onto 10% (vol/vol) polyacrylamide gels. After SDS-PAGE, proteins were transferred onto nitrocellulose membrane (Protran, Schleicher & Schuell, Germany) and detected using a rabbit anti-MMP-9 (Chemicon, El Segundo, CA) at 1:1000 dilution, a matching secondary antibody (Jackson ImmunoResearch, Cambridgeshire, UK), and a chemiluminescent substrate (Pierce, Rockford, IL), according to the manufacturer's instructions. The antibody binding was imaged on X-OMAT film (Eastman Kodak, UK). To account for the inter-blot variations in MMP immunoreactivity, internal standard (conditioned medium of HT-1080 human fibrosarcoma cells, recombinant MMP-2 and MMP-9) was used, and the results were standardized by comparison with standard [18], using a calibrated densitometer (GS-800, Bio-Rad) and Quantity One Quantitation analysis software (Version 4, BioRad). Protein expression of each band was measured in terms of ODu/mm. Western blotting with monoclonal anti-β-actin (Sigma Chemical Co.) was performed as internal control.

### MPO assay

For the assay of MPO activity, colonic specimens were homogenized in 2 ml phosphate-buffered saline using a Tissue Tearor (model 985-370, Biospec, Racine, WI) and centrifuged [19]. The pellets were again homogenized in an equivalent volume of phosphate buffer (50 mM, pH 6) containing 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co.) and 5 mM EDTA, sonicated three times for 30 s each time (Labasonic 2000, Braun, Germany), 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 peroxide/min at 25°C.

### Assessment of colonic damage

Two observers, who were unaware of the treatment applied, separately scored macroscopic lesions and then averaged them [20, 21]. The macroscopic score was obtained by assessment of colonic strictures, adhesions to surrounding tissues, mucosal ulcerations, and wall-thickening, according to the criteria shown in **Table 1**. Samples were processed for histological examination using routine techniques before embedding in paraffin. Sections were obtained from areas showing macroscopic damage, stained with hematoxylin and eosin, and coded for blind examination by two pathologists. Both pathologists examined and scored all sections according to the presence of ulcerations, degree of inflammation, depth of the lesions, and fibrosis (Table 1).

### Immunohistochemistry of MMP-9

Immunohistochemistry was performed as described previously [9]. Briefly, samples were frozen in liquid nitrogen and embedded in Tissue Tek mountant (BDH Merck Ltd., Poole, UK). Sections of 7 μm were cut on a cryostat and fixed with acetone. The sections were probed with rabbit and mouse monoclonal anti-MMP-9 (1:150 dilution) and detected with 1:500 dilution of sheep

TABLE 1. Morphologic Criteria for Assessment of Colonic Damage<sup>a</sup>

Macroscopic score		
Adhesions	None	0
	Minimal	1
	Involving several bowel loops	2
Strictures	None	0
	Mild	2
	Severe, proximal dilatation	3
Ulcers	None	0
	Linear ulceration <1 cm length	1
	Two linear ulcers <1 cm	2
	More sites of ulceration or one large ulcer >1 cm	3
Wall thickness	Less than 1 mm	0
	1–3 mm	1
	More than 3 mm	2
	Maximum possible score	10
Histological score		
Ulceration	No ulcers	0
	Small ulcers <3 mm	1
	Large ulcers >3 mm	2
Inflammation	None	0
	Mild	1
	Moderate	2
	Severe	3
Depth of lesion	None	0
	Submucosa	1
	<i>Muscularis propria</i>	2
	Serosa	3
Fibrosis	None	0
	Mild	1
	Severe	2
		Maximum possible score

<sup>a</sup> Total score was obtained by summation of macroscopic and histological scores.

anti-rabbit or sheep anti-mouse immunoglobulin G fluorescein isothiocyanate (FITC). Sections were counterstained with 5  $\mu\text{mol/L}$  propidium iodide and examined under a fluorescent microscope. Control sections were stained without the primary antibody but with the secondary FITC [9].

## Effects of MMP Inhibition

To determine whether up-regulation of MMP-9 modulates the degree of TNBS-induced colitis, we performed additional *in vivo* experiments inducing early or delayed inhibition of MMPs with CGS-27023-A. The compound CGS-27023-A [*N*-hydroxy-2<sup>®</sup>-(4-methoxysulfonyl)(3-picoly)-(amino-3-methylbutaneamidine-hydrochloride monohydrate)] is a potent, orally available, nonpeptidic, synthetic inhibitor of MMPs activity [12]. Novartis (Verna, Switzerland) supplied a sample of CGS-27023-A for the purpose of this study.

First, the activity of CGS-27023-A as an inhibitor of MMP activity was assessed *in vitro* by duplicated zymographies using colonic homogenates from rats subjected to TNBS during 10 days. After electrophoresis, both gels were subjected to development buffer, and CGS-27023-A (20 ng/ $\mu\text{L}$ ) was added to one of them before overnight incubation. After 18 h development, gelatinolytic bands were red, comparing their size in the presence or absence of MMP inhibitor.

Two sets of experiments were performed to assess the effect of MMP inhibition on TNBS-induced intestinal injury. First, TNBS transmural colitis was induced in two groups of 15 rats each. One group comprised 15 rats, which received CGS-27023-A by oral gavage at 20 mg/Kg/day from day 0 to day 10 after induction of colitis (early MMP inhibition), whereas the control group

received oral saline. In a second set of experiments, again, TNBS transmural colitis was induced in two other groups of 15 rats each, but the treatment group received the MMP inhibitor from day 11 to day 21 after colonic enema instillation (delayed MMP inhibition), whereas the control group received oral vehicle.

In early and delayed treatment groups, body weight was obtained routinely every second day. On day 21, rats were killed by cervical dislocation, and colons were removed, opened longitudinally, and divided in two specimen parts. One specimen was homogenized and stored at  $-20^{\circ}\text{C}$  for MPO activity, whereas the second specimen was used for microscopic assessment.

In addition, to assess the activity of CGS-27023-A as a MMP inhibitor *in vivo*, transmural colitis was induced in two groups of four rats each, and another four rats received saline enema. One colitic group received the MMP inhibitor (CGS-27023-A, 20 mg/Kg/day) by oral gavage from day 0 to day 10 after induction of colitis, whereas another colitic group received saline. On day 10, all rats were killed by cervical dislocation, and colons were removed, rinsed with saline, homogenized, and stored at  $-20^{\circ}\text{C}$  for MPO and MMP activities.

## RESULTS

### TNBS induction of colitis

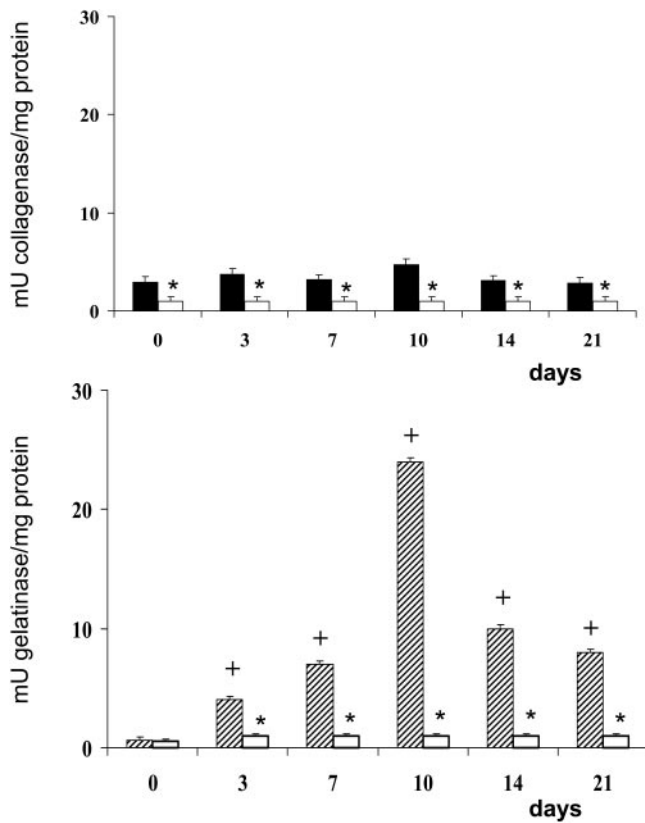
Rectal administration of TNBS induced diarrhea and loss of weight in all rats. Gross tissue damage observation and examination of stained tissue sections revealed that administration of TNBS at this concentration resulted in mucosal ulcerations with granulation tissue at the base and severe transmural inflammatory response. In the acute phase (until day 10), the inflammatory infiltrate consisted predominantly in polymorphonuclear leukocytes. In the chronic phase, in some cases, small granulomas were observed in the submucosa and serosa. The inflammatory infiltrate consisted mainly of mononuclear cells, and transmural fibrosis was observed in most of cases. However, rectal administration of saline enema did not have any effect in macroscopic and histological examination of the colonic tissue.

### Total collagenase and gelatinase activity

**Figure 1** shows total collagenase and gelatinase activity in colonic homogenates of rats before (day 0) and at different time-points following the administration of TNBS (days 3–21). A significant up-regulation in tissue gelatinase concentration was observed on day 3 after the induction of colitis, but peak levels were reached on days 7 and 10 after TNBS instillation. In contrast, collagenase activity in homogenates of colonic tissues remained unaltered by TNBS administration. The MMP activity was inhibited completely when 1, 10-phenantroline was added to the buffer.

### Up-regulation of MMP-9 activity in TNBS colitis

**Figure 2A** shows zymograms of colonic homogenates from control and colitic rats treated with TNBS. 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. Induction of colitis resulted in the appearance of pro-MMP-9 in all animals in addition to pro-MMP-2 activity. Furthermore, on days 7 and 10 after induction of colitis, the active form of MMP-2 was increased in some specimens but did not reach any statistical significance. Both gelatinase activities were eliminated by incubating the gels with 1 mmol/L EDTA or 1,



**Fig. 1.** Effect of phenantroline on total collagenase and gelatinase activity. Total collagenase and gelatinase activity in colonic homogenates from rats before (day 0,  $n=5$ ) and at different time-points following the administration of TNBS ( $n=7$  each group). The MMP activity was markedly attenuated. \*,  $P < 0.01$ , by the MMP inhibitor phenantroline; +,  $P < 0.05$ , significantly higher than gelatinase activity samples on day 0

10-phenantroline and were re-established by adding 5 mmol/L  $Ca^{2+}$  and  $Zn^{2+}$ . However, these gelatinolytic bands were not modified by adding aprotinin or leupeptin into the gels. The densitometric analysis (Fig. 2B) showed a significant up-regulation of pro-MMP-9, especially on Days 7 and 10 after induction of colitis compared with control rats. The time course of the rise of MMP-9 and total gelatinase activity was essentially concordant.

### MMP-9 expression in TNBS colitis

**Figure 3A** shows the Western blot analysis of pro-MMP9 on colonic homogenates from controls and rats with colitis induced by TNBS. Colitis resulted in the appearance of pro-MMP-9, as shown by the immunoreactive band migrating at 92 kDa. The densitometric analysis (Fig. 3B) demonstrated a significant up-regulation in tissue pro-MMP-9 concentration on day 3 after induction of colitis, but again, peak levels were reached on days 7 and 10 after TNBS instillation.

### Cellular origin of MMP-9 in inflamed tissues

TNBS enema instillation resulted in a significant increase in MPO activity in colitic animals in comparison with control rats. Furthermore, MPO activity in colitic samples reached its maximum on days 7 and 10 after induction of colitis (**Fig. 4A**). The

increase in MPO activity was essentially concordant with the increase of pro-MMP-9 activity and expression.

To assess whether PMN were activated to release MMP-9 during TNBS colitis, we isolated peripheral PMN ( $5 \times 10^6$  ml) from normal and colitic rats 10 days after induction of colitis. Consistent with mucosal samples, a band of gelatinolysis for homogenates of purified neutrophils was found in colitic rats. A band migrating at 92 kDa by zymography (Fig. 4B) or Western blot (Fig. 4C) confirmed that this enzyme was pro-MMP-9. By contrast, MMP-2 was not detected by either technique in these samples. In addition, this gelatinolytic band was inhibited by CGS-27023-A *in vitro*. In the supernatant of an equivalent number of PMN isolated from healthy animals, no gelatinolytic band was observed.

As shown by immunofluorescent staining (**Fig. 5**), low levels of MMP-9 were detected in areas of noninvolved colitis and normal colonic tissue, whereas there was evidence for a transmural MMP-9 deposit in the inflamed samples. This finding was related to an increased inflammatory cell infiltrate throughout the intestinal wall, mostly located at the lamina propria and predominantly subepithelial (Fig. 5).

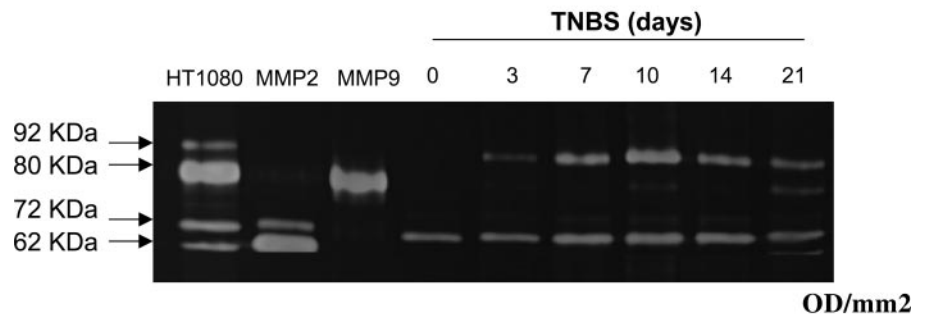
### Inhibition of MMP activity by CGS-27023-A

*In vitro* zymographic studies showed that 20 mg/l CGS-27023-A abolished MMP activity in colitic tissue samples on day 10 after the induction of colitis (data not shown). In addition, CGS-27023-A at the same concentration was able to inhibit pro-MMP-9 activity in the supernatants of peripheral PMN of colitic rats.

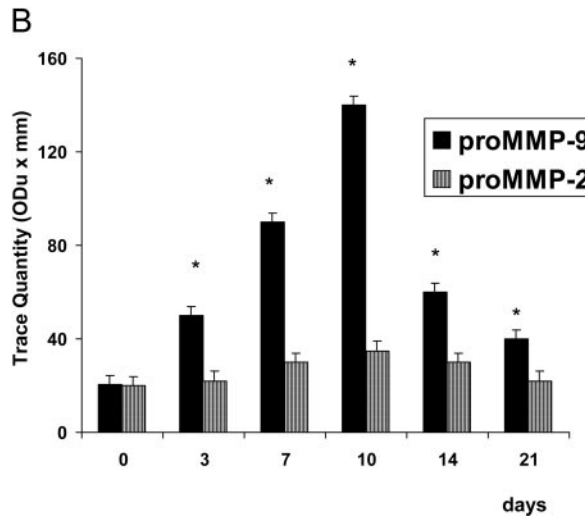
The effect of MMP inhibition on morphological lesion scores of rats with chronic TNBS colitis treated with CGS-27023-A is shown in **Figure 6**. In the study corresponding to early treatment with CGS-27023-A, one rat from the placebo group and another one from the experimental group died immediately after the induction of damage. In addition, one rat from the placebo group died during the follow-up, so that by the end of the study, there were 13 and 14 surviving rats in each group. By day 21, normal growth rates were resumed, and rats treated with the CGS 27023-A were statistically heavier ( $280 \pm 10$  gr) than placebo-treated rats ( $25 \pm 14$  gr). Rats receiving CGS 27023-A from day 0 after induction of colitis showed a significant decrease ( $P < 0.05$ ) on macroscopic and histological scores (Fig. 6A). Adhesions and thickening of the wall were significantly lower ( $P < 0.05$ ) than in the placebo group. Likewise, histological scores were significantly lower ( $P < 0.05$ ) in rats that received the MMP inhibitor (Fig. 6A).

In the study in which the effect of delayed treatment with CGS-27023-A was evaluated, two rats from the placebo group and another one from the treatment group died before treatments began. Thereafter, one rat from the placebo group died during the follow-up, and at the end of the study, there were 12 surviving rats in the placebo group and 14 in the treatment group. Body weight was not statistically different between rats treated with saline ( $260 \pm 13$  gr) and those receiving delayed therapy with CGS 27023-A ( $266 \pm 20$  gr). In addition, morphological and histological scores were similar between the two experimental groups (Fig. 6B). MPO activity values in TNBS-colitis rats receiving delayed treatment with CGS 27023-A

A



**Fig. 2.** Activity of the pro-MMP-9 and pro-MMP-2 forms in TNBS-induced colitis. (A) Representative zymography of tissue samples before (day 0, n=5) and at different time-points following the induction of colitis (n=7 each group). (B) Densitometric analysis showed a significant up-regulation of pro-MMP-9 activity in TNBS colitis, whereas pro-MMP-2 remained unaltered (\*,  $P < 0.05$ , vs. day 0).



were not significantly different from rats treated with placebo (data not shown).

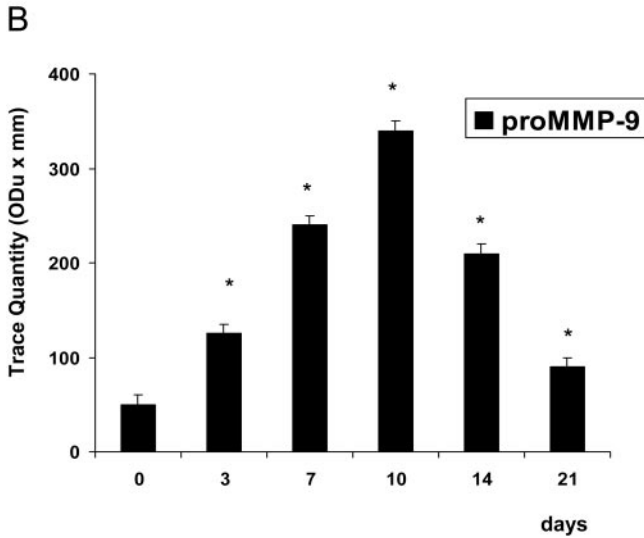
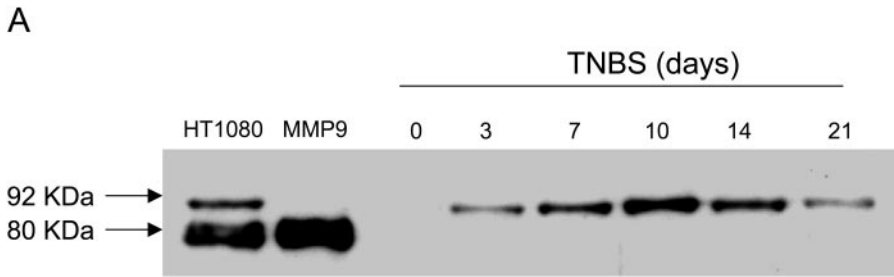
As MMP-9 activity reached the highest level on day 10 after induction of colitis, we tested the effect of CGS-27023-A on tissue MMP-9 activity induced by TNBS instillation. **Figure 7A** shows the *in vivo* inhibition of MMP-9, but not of MMP-2, on homogenates of colonic tissue in rats treated with CGS-27023-A. In addition, MPO activity was significantly lower in rats receiving the MMP inhibitor in comparison with the placebo group (Fig. 7B).

## DISCUSSION

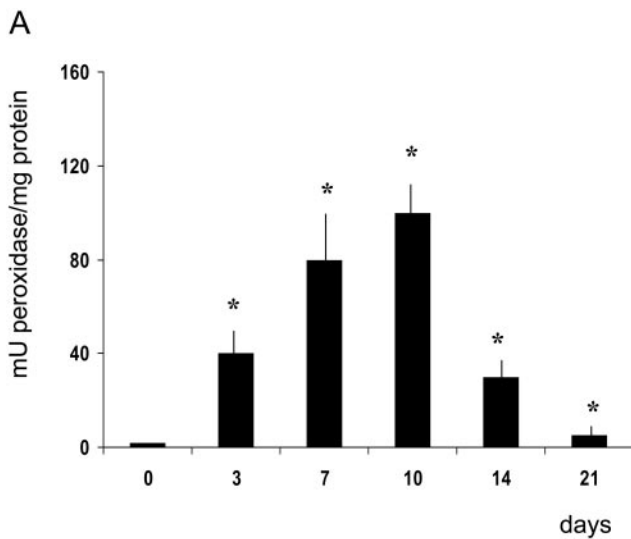
Several lines of evidence have suggested that MMPs play an important role in the pathogenesis of IBD [22–24]. First, it has been demonstrated that the activation of T cells in explant cultures of fetal human intestine caused mucosal damage mediated by these enzymes. In fact, MMPs promoted severe tissue injury when added directly to the explants, whereas mucosal damage was prevented with selective MMP inhibitors [22]. Second, up-regulation of intestinal MMPs has been associated with intestinal inflammation and tissue degradation in patients with CD [9–12]. The present study shows that up-regulation of MMP-9 modulates transmural intestinal injury in rats with chronic colitis induced by TNBS instillation. We found that TNBS-induced colitis resulted in an increase of MMP-9 activ-

ity and expression in all specimens, especially on days 7 and 10 after TNBS enema instillation. However, zymographic analysis showed no significant difference in MMP-2 activity between control and colitic rats. Previous studies have shown that gene transcription of MMP-9 is inducible and that the promoter region is highly responsive to most growth factors and cytokines in several cells. By contrast, MMP-2 is expressed constitutively by most cells and appears to be moderately induced or repressed [25, 26]. Our results are consistent with a previous study of an experimental model of colitis induced in immunodeficient mice by transfer CD4<sup>+</sup> lymphocytes [27]. In this study, transmural inflammation was associated with up-regulation of MMP-9 activity in colonic tissues. In addition, we have shown previously that MMP-9 activity is increased dramatically in intestinal samples in a rat model of dextran sulfate sodium-induced colitis [28].

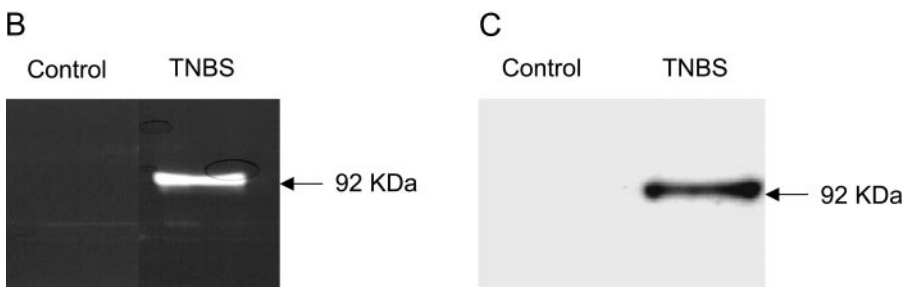
In the present study, consistent with colonic MMP-9 activity, there was a concordant increase in MPO activity, which represents the amount of neutrophils trapped into intestinal tissue. Hence, to elucidate the source of MMP-9 found in colitic samples, we next studied peripheral PMN isolated from control and colitic rats, as they have been shown to release MMP-9 [17, 29]. Zymographic analysis showed an increase of pro-MMP-9 activity in the supernatant of peripheral neutrophils isolated from colitic rats compared with healthy control animals. However, MMP-2 activity was not found in peripheral neutrophils isolated from colitic rats in contrast to the consti-



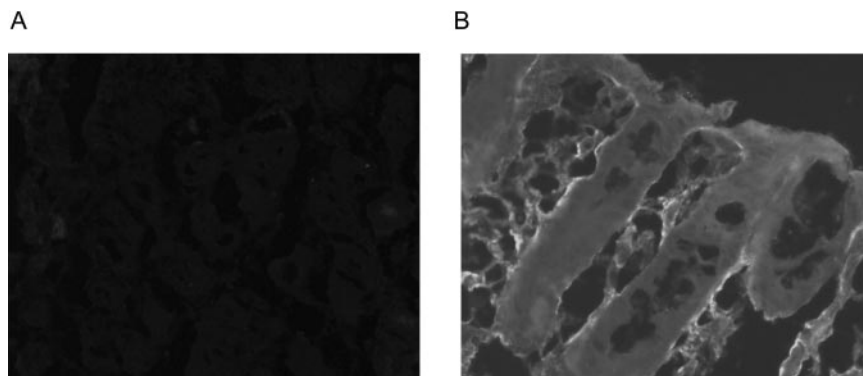
**Fig. 3.** Expression of pro-MMP9 in TNBS-induced colitis. (A) Representative Western blot of pro-MMP-9 of colonic samples before (day 0, n=5) and at different time-points following the induction of colitis (n=7 each group). (B) Densitometric analysis showed a significant increase in pro-MMP-9 protein in rats with colitis induced by TNBS (\*,  $P < 0.05$ , vs. day 0).



**Fig. 4.** MPO activity and neutrophil release of pro-MMP-9. (A) MPO activity in homogenates of colonic tissue before (day 0, n=5) and at sequential times in the course of TNBS-induced colitis (n=7 each group). MPO levels reached a peak at day 10 after TNBS administration. (B) Zymography in peripheral neutrophils showing pro-MMP-9 activity in controls (n=5) and colitic rats (n=5) on day 10 following the administration of TNBS. (C) Western blot analysis of pro-MMP-9 expression in polymorphonuclear neutrophils (PMN) from controls (n=5) and rats with TNBS-induced colitis (n=5) on day 10 after the induction of colitis.



**Fig. 5.** Immunohistochemistry of MMP-9 in colonic tissue using monoclonal anti-MMP-9 antibody (n=4 each group). Low levels of MMP-9 were detected in noninvolved colonic tissue (A), whereas there was a strongly positive, transmural staining in TNBS-induced colitis, mostly in the lamina propria (intense green fluorescence;  $\times 400$ ) (B).

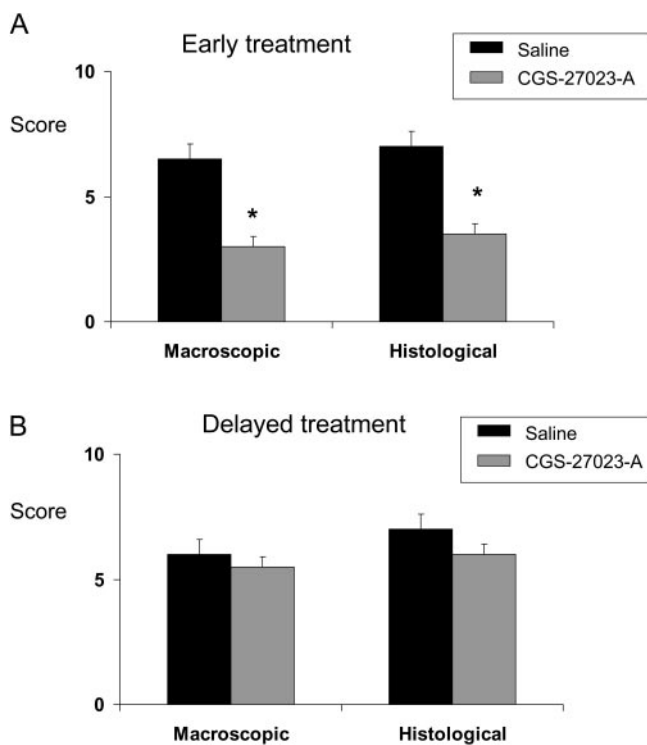


tutive expression of this enzyme in colonic tissues. These results are in agreement with other groups, which demonstrated the inability of PMN to secrete MMP-2 [17]. In fact, in an experimental rat model of acute pancreatitis-associated lung injury, MMP-9 protein was expressed highly in lungs and supernatants of PMN cultures from animals with severe pancreatitis, suggesting an important role for this enzyme in the pathogenesis of lung injury in that experimental model [17]. Furthermore, in our study, the immunofluorescent staining of frozen sections showed a large amount of transmural MMP-9, consistent with the distribution of infiltrating PMN in the injured tissues. These results are in concordance with a previous study in humans, which showed a large amount of neutrophils, staining positive for MMP-9 throughout the intestinal

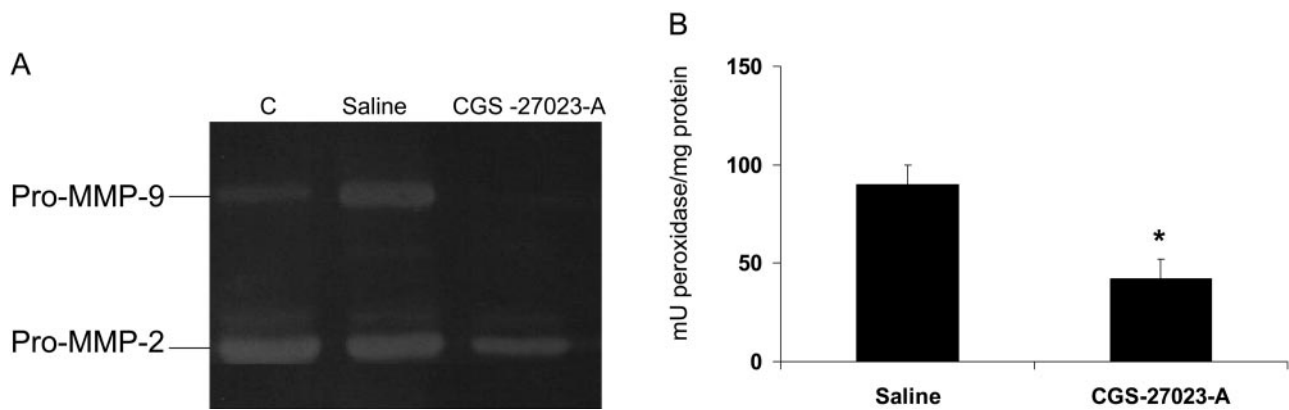
wall in CD patients [30]. In addition, MMP-9 seems to be the main protease responsible for the accelerated breakdown of ECM in that pathological condition, as it was demonstrated that this enzyme is the most abundantly MMP-expressed in colonic tissues from patients with CD compared with controls [9]. Therefore, taken together, PMN could be the main source of MMP-9 in TNBS-induced colitis, where activation of this enzyme could lead to excess degradation of ECM and loss of tissue organization.

To study whether the up-regulation of MMP-9 may modulate the intestinal injury in TNBS-induced colitis, we next tested the effect of a broad-spectrum MMP inhibitor, CGS-27023-A [31], on tissue damage. This compound has been tested successfully in several pathological conditions in vitro and in vivo [28, 32, 33]. In the current study, the MMP inhibitor effectively induced histopathological remission of colonic inflammatory lesions when started at the acute phase of the colitis. In addition, the administration of CGS-27023-A was associated with increased body weight gain in rats subjected to the early treatment as compared with control rats. This effect correlated well with pro-MMP-9 inhibition; however, the compound was not able to inhibit pro-MMP-2 activity. These results are consistent with previous studies, where broad-spectrum MMP inhibitors were able to inhibit the activation of MMP-2, but they did not exert any effect in the pro-form [32, 34]. By contrast, CGS-27023-A did not have any histological effect when the administration started beyond the acute phase of the disease. These data suggest that MMPs may be a key factor on transmural intestinal damage occurring during the initial stage of TNBS-induced colitis and are consistent with our previous study in a rat model of chronic colonic transmural inflammation induced by *Bacteroides fragilis* [35]. In that study, MMP inhibition prevented intestinal injury, suggesting that these enzymes were responsible for transmural inflammation associated with bacterial migration through the intestinal wall.

It is interesting that we also found that the administration of CGS-27023-A decreased tissue levels of MPO on day 10 after the induction of colitis, suggesting that this compound affected the recruitment of MPO-expressing inflammatory cells such as PMN into inflamed tissue at the early phase of the disease. These findings suggest that MMP-9 released by PMN could be an important protease for transmigration of these cells across a basement membrane, such as Delclaux et al. [36] found in a previous in vitro work. In that study, TIMP-1 was found to inhibit trans-basement membrane PMN migration without af-



**Fig. 6.** Effect of MMP inhibition on TNBS-induced colitis. (A) CGS-27023-A treatment significantly reduced the macroscopic and histological scores when started on day 0 (n=15 each group; early treatment; \*,  $P < 0.05$ , vs. placebo). (B) The MMP inhibitor failed to improve colitic lesions when started on day 11 (n=15 each group; delayed treatment). All rats were killed on day 21 after TNBS instillation.



**Fig. 7.** Effect of CGS-27023-A on MMPs and MPO activity on day 10 after the induction of colitis ( $n=4$  each group). (A) Representative zymographic analysis of MMP-2 and MMP-9 in colonic tissues of control (C) rats and in colitic rats receiving saline or CGS-27023-A. (B) MPO activity was markedly attenuated in colitic rats treated with CGS-27023-A ( $P<0.05$  vs. placebo).

fecting PMN chemiotaxis or chemokinesis. By contrast, we did not find any significant difference in MPO activity on day 21 after the induction of colitis between treatment and placebo groups. This result could be explained, as PMN are replaced mainly by mononuclear cells at the final stage of the TNBS experimental model, and therefore, the tissular MPO activity returned to baseline levels on day 21 after enema instillation. Hence, to find a statistically significant difference between treated and untreated groups could be extremely difficult, as we have found previously [21]. However, further investigation is needed to clarify the precise role of neutrophilic MMP-9 in this experimental model.

In summary, the present study showed that an increased activity and expression of peripheral and intestinal MMP-9 are associated positively with transmural colonic injury in rats with TNBS-induced colitis. The inhibition of MMP activity prevented colonic injury at the early phase of this experimental model of colitis and raises the possibility of a new therapeutic approach to human CD.

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