Pathol. Oncol. Res. (2012) 18:85–91
DOI 10.1007/s12253-011-9420-9

RESEARCH

The Behavior of Matrix Metalloproteinase-9 in Lymphocytic Colitis, Collagenous Colitis and Ulcerative Colitis

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Received: 7 May 2010 / Accepted: 26 May 2011 / Published online: 16 June 2011

Abstract Matrix metalloproteinases play an important role in extracellular matrix remodelling. It has been proposed that matrix metalloproteinase-9 (MMP-9) is involved in epithelial damage in ulcerative colitis (UC). However, to our knowledge, no data are available in terms of MMP-9 expression in microscopic colitis. Determination of mucosal protein expression levels of MMP-9 in lymphocytic colitis (LC), collagenous colitis (CC) and UC. MMP-9 immunohistochemical expressions were analyzed in paraffin-embedded tissue samples by immunohistochemistry including patients with LC, CC, UC, active diverticulitis, inactive diverticular disease and healthy control subjects. UC was also subgrouped according to the severity of inflammation. Immunostaining was determined semiquantitatively. Independent colonic biopsies from healthy and severe UC cases were used for gene expression analyses. For further comparison MMP-9 serum antigen levels were also determined in patients with UC and control patients without macroscopic or microscopic changes during colonoscopy. MMP-9 mucosal expression was significantly higher in UC (26.7±19.5%) compared to LC (6.6±9.3%), CC (6.4±7.6%), active diverticulitis (5.33±2.4%), inactive diverticular disease (5.0±2.2%) and controls (6.3±2.6%) ($P<0.001$). The immunohistochemical expression of MMP-9 in LC and CC was similar as compared to controls. MMP-9 expression was significantly higher in each inflammatory group of UC compared to controls (mild: 11.0±2.8%, moderate: 23.9±3.7%, severe UC: 52.6±3.9% and 6.3±2.6%, respectively, $P<0.005$). The gene expression microarray data and RT-PCR results demonstrated a significantly higher expression of MMP-9 in severely active UC compared to healthy controls ($P<0.001$). Significantly higher MMP-9 serum antigen concentrations were observed in UC patients compared with the control group ($P<0.05$). MMP-9 seems to play no role in the inflammatory process of LC and CC. In contrast, the mucosal up-regulation of MMP-9 correlated with the severity of inflammation in UC. The increased MMP-9 expression could contribute to the severity of mucosal damage in active UC.

Keywords Matrix metalloproteinase · Ulcerative colitis · Lymphocytic colitis · Collagenous colitis · Immunohistochemistry · Inflammation

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Introduction

Inflammatory bowel disease (IBD), i.e. Crohn's disease (CD) and ulcerative colitis (UC), are characterized by inflammation and ulceration of the gastrointestinal tract [1]. In the pathophysiological process of IBD, a variety of inflammatory mediators, such as proteolytic enzymes, cytokines and growth factors, and many kind of cells, like leukocytes and stromal cells, are implicated in the tissue injury and healing processes [2].

Proteolytic enzymes are thought to be major contributors to the breakdown and reconstitution of extracellular matrix (ECM) in physiological processes and in pathological conditions, including destructive diseases and tumor progression [3]. We have previously demonstrated that proteo-

lytic enzymes, such as cysteine and serine proteases, may have a role not only in the process of gastrointestinal (GI) cancer invasion, or in the progression of gastrointestinal precancerous changes into cancer, but also they are widely distributed in gastrointestinal tissues and they have been implicated in processes of GI inflammation, tissue remodelling, angiogenesis and wound healing [4–8].

Matrix metalloproteinases (MMPs) play an important role in the process of tissue remodelling and destruction during inflammation [9–12]. We and others have reported that type IV collagenase, matrix metalloproteinase-9 (MMP-9) (gelatinase B) to be especially important in the process of tumor invasion and metastasis [13, 14], but also in the inflammatory and remodelling processes in IBD [15–19].

Microscopic colitis (MC) is characterized by macroscopically normal mucosa during colonoscopy, where diagnostic histopathological features are seen on microscopic examination. MC includes collagenous colitis (CC) and lymphocytic colitis (LC) [20]. The cause of MC is unknown and probably the etiology is multifactorial. The suspected relationship between immune dysregulation and MC has led to the question of whether MC is a pre-IBD entity. There are very few data supporting this hypothesis [21].

To our knowledge, MMP-9 expression has not been determined in correlation with the severity of inflammation in UC. To date there are no data available in terms of MMP-9 expression in MC. Therefore, the aim of the present study was to determine the mucosal protein expression levels of MMP-9 in LC, CC and UC. We further investigated the behavior of MMP-9 expression in relation to the severity of mucosal inflammation in patients with UC using immunohistochemical analyses. As an independent set of samples biopsies from healthy normal controls and severely active UC patients were taken from the colon during colonoscopy and gene expression analyses were done. For further comparison MMP-9 serum antigen levels were also determined in patients with UC and control patients.

Methods

MMP-9 Immunohistochemistry

Tissue specimens were obtained endoscopically from in- and outpatients at the 2nd Department of Medicine, Semmelweis University Budapest. After informed consent, during the routine colonoscopies, colorectal biopsy samples were taken from the most affected part of the colon from LC (n=64), CC (n=28) and UC patients (n=34). UC was sub-grouped according to the severity of inflammation (mild: n=11, moderate: n=11, and severe UC: n=12). Colorectal biopsies during colonoscopy from patients with active diverticulitis (n=8) and inactive diverticular disease (n=8) were obtained for further comparison. Colonoscopic biopsies from patients with irritable bowel syndrome (IBS) without any histological changes were used as healthy controls (n=10). The endoscopic specimens were fixed in formalin and embedded in paraffin wax, sliced serial step sections of 4 μm thickness.

To assess the location of MMP-9 within the intestinal tissues, immunohistochemical staining for the MMP-9 was performed as described previously [13]. Briefly, after dewaxing in xylene and rehydration through graded ethanol, endogenous peroxidase activity was blocked by incubation for 30 min at room temperature in 3% hydrogen peroxide. After PBS washing, non-specific blocking was done with 1% BSA-PBS solution for 10 min at room temperature. Then the slides were incubated with optimally diluted monoclonal anti-human MMP-9 antibody (Clone: 36020.111, R&D Systems) at 37°C for 60 min in a humidified chamber. After washing them 3 times in PBS, signal conversion was carried out with the LSAB2 system (DAKO) as described in the manual. Haematoxylin co-staining was done. Tissue sections from human ovarial carcinoma were used as positive and negative controls.

Determination of the Degree of Inflammation in UC

The degree of inflammation was determined semiquantitatively by experienced pathologist, as previously described [22]. The activity of inflammation was mild if low neutrophil and eosinophil infiltration was found in the epithelial layer and in lamina propria, most of the crypts were straight, and the integrity of the epithelium was retained. Severely active inflammation was characterized by extensive neutrophil and eosinophil infiltration in the epithelial layer and in lamina propria, severe crypt distortion, extended decrease of crypt density, transparent, villous epithelial surface. The inflammation was moderately active if a transitional state between mild and severe inflammation was found.

Counting

None of the control sections exhibited immunoreactivity. Immunostaining was determined as described previously [22]. Essentially, two independent investigators without knowledge of the clinical outcomes evaluated the degree of immunohistochemical staining intensity. There was less than 5% variance between the results of two counts. Mucosal immunoreactivity was determined in the crypt epithelial cells and in the lamina propria, than taking the latter two together the cumulative expression described the whole mucosa. Axially, at least 500 (mainly 800) crypt

epithelial cells within well-oriented crypts were counted in each samples using light microscope. In the lamina propria five fields of view at 40× magnification were counted in each sample. Labeling index (%) was described as the ratio of the positive cell number and the number of counted cells.

Gene Expression Analysis

As an independent set of samples, for gene expression analysis, biopsies from 11 healthy normal controls and 12 severely active UC patients were taken from the colon during colonoscopy, and stored in RNALater Reagent (Qiagen Inc., US) at −80°C. Total RNA was extracted and gene expression analyses were done.

The gene expression microarray analysis was performed as described previously [23].

Taqman RT-PCR

MMP-9 (TaqMan probe ID: Hs00957562_m1), HGFR (TaqMan probe ID: Hs00179845_m1) TaqMan real-time PCR was used to measure the mRNA expression of the observed parameter using an Applied Biosystems Micro Fluidic Card System. The measurements were performed using an ABI PRISM® 7900HT Sequence Detection System as described in the products User Guide (http://www.appliedbiosystems.com, CA, USA). The total cycle number was 45.

Enzyme-Linked Immunoassay ELISA

For further comparison MMP-9 serum antigen levels were also determined in five patients with (UC) without dysplasia as confirmed by colonoscopy and biopsy (four males and one female, mean age: 51.2 years (range 33–76 years), and seven control patients without macroscopic or microscopic changes (three males and four females, mean age: 48.0 years (range 37–57 years).

Serum samples were collected from patients with UC at the time of clinical diagnosis. Blood samples were collected from resting patients after a 12 h fasting between 8:00 and 10:00 a.m. Blood was drawn from a cubital vein with minimal venous occlusion directly into plastic tubes prepared with sodium citrate (0.1 M final concentration) as an anticoagulant. The samples were stored at −70°C until analysis.

Serum antigen levels for MMP-9 were measured by using the enzyme-linked immunoassay (ELISA) method as follows: briefly, MMP-9 immunoassay is a solid-phase ELISA based on a sandwich principle (Human MMP-9, R&D Systems, DMP900). Absolute quantities of MMP-9 antigens in the serum samples were calculated from an 8-point standard curve of MMP-9 (0–10 ng/ml). The lowest detectable concentrations were estimated at $\cong 0.312$ng/ml.

Statistical Analysis

Statistical analysis with one-way ANOVA (analysis of variance) and LSD (least significant difference) test were performed by the Statistica for Windows 4.3 program package. P value of <0.05 was considered significant.

In case of Taqman RT-PCR, for data analysis the SDS 2.2 software was used. The extracted delta Ct values (which represent the expression normalized to the ribosomal 18S expression) were grouped according to the histologic groups. Then the Student's t-test was performed to compare the expression values between groups.

Correlation analysis was done between the calculated real time RT-PCR expression values (total cycle number – delta CT) and the immunohistochemical expression values in case of normal samples and severely active UC samples. The real time RT-PCR values were compared to both crypt epithelial expression values and the expression values of the lamina propria (if the protein expression values were measurable).

Ethical Consideration

All routine colonic biopsy specimens during colonoscopy from the patients were taken after informed consent and ethical permission was obtained for participation in the study.

Results

MMP-9 Immunohistochemistry

The degree of immunohistochemical expression of MMP-9 in LC, CC, UC, active diverticulitis, inactive diverticular disease and in normal intestinal tissue is shown in Table 1. MMP-9 mucosal expression was significantly higher in UC compared to LC, CC, active diverticulitis, inactive diverticular disease and controls ($P<0.001$). No significant differences were observed regarding MMP-9 mucosal expression between LC and CC compared to active diverticulitis, inactive diverticular disease or controls. Finally, no significant differences were detected between active diverticulitis compared to inactive diverticular disease and controls (Table 1.)

The expression of MMP-9 in UC increased in line with the severity of inflammation (Table 2.). MMP-9 expression was significantly higher in each inflammatory group of UC compared to controls ($P<0.005$).

Table 1 Matrix metalloproteinase-9 (MMP-9) immunohistochemical expression in ulcerative colitis, lymphocytic colitis, collagenous colitis, active diverticulitis, inactive diverticular disease and in normal colonic tissue. (Data are means ± standard deviations of the labeling indices (%); Control: Normal colonic tissue)

Histology	Immunohistochemical expression of MMP-9
Ulcerative colitis (n=34)	26.7±19.5
Lymphocytic colitis (n=64)	6.6±9.3
Collagenous colitis (n=28)	6.4±7.6
Active diverticulitis (n=8)	5.3±2.4
Inactive diverticular disease (n=8)	5.0±2.2
Control (n=10)	6.25±2.56
ANOVA one-way	P<0.001

MMP-9 was expressed mainly within the cytoplasm and cytoplasmic membranes of the colonic epithelial cells. The prominent location of MMP-9 was in the submucosa. A pronounced positive staining for MMP-9 in polymorphonuclear leucocytes (PMNL) was found. In addition, a weak positive reaction of MMP-9 was observed in lamina propria, which was found to be increased with severity of the inflammation in UC tissues. Predominantly in inflamed area, occasionally macrophages positive for MMP-9 were also found. In normal tissues no other major cells showed MMP-9 immunoreactivity (Fig. 1).

Gene Expression Analyses

As an independent set of samples MMP-9 gene expression analyses were performed. The expression values (calculated numbers without a measure) in severely active UC (n=12) were compared to the expression values of healthy normal controls (n=11).

Table 2 Matrix metalloproteinase-9 (MMP-9) immunohistochemical expression in normal colonic tissue and in ulcerative colitis. (Data are means ± standard deviations of the labeling indices (%); Control: Normal colonic tissue; UC: ulcerative colitis)

Histology	Immunohistochemical expression of MMP-9
Control (n=10)	6.25±2.56
Mild UC (n=11)	11.04±2.78
Moderate UC (n=11)	23.96±3.67[c]
Severe UC (n=12)	52.63±3.94[a,b]
ANOVA one-way	P<0.005

Statistics: [a] P<0,001 vs. Control and mild UC

[b] P<0,05 vs. Moderate UC

[c] P<0,05 vs. Control

MMP-9 expression was significantly higher in severe UC compared to healthy controls (10.69±1.02 vs. 7.15± 0.88, P<0.001).

Taqman RT-PCR

For confirmation of immunohistochemical and gene expression analyses results MMP-9 TaqMan real-time RT-PCR analysis was performed. The expression values in active UC were compared to the expression values of healthy ones. The expression of MMP-9 was 3.62 times higher in active UC than in healthy colon (2^(−ddCt): −3.616; p<0.05).

Correlation Analysis

In case of MMP-9, significantly positive correlation was found between the cumulative protein expression values (expression in crypt epithelium and lamina propria together) and the calculated mRNA expression values in the analyzed normal and active UC samples (r>0.95). Lamina proprial MMP-9 protein expression also showed significant positive correlation to the calculated mRNA expression values in the same disease groups (r>0.95).

For gene expression analyses we used biopsy samples from macroscopically healthy and severely active UC mucosa, but not mildly and moderately active inflammatory samples. One explanation of this that the most significant expression differences between the healthy and the severely inflamed UC samples were expected. Furthermore, macroscopically it is impossible to make sharp differences between mild and moderate inflammation during colonoscopy.

ELISA

Serum antigen concentrations for MMP-9 in patients with UC and controls, expressed in ng/ml, are shown in Fig. 2. Significantly higher MMP-9 serum antigen concentrations were observed in UC patients compared with controls (743.34±138.5 vs 451.1±67.6 ng/ml, P<0.05).

Discussion

Proteolytic enzymes may have a role not only in the process of GI cancer invasion, or in the progression of precancerous changes into cancer, but they have also been implicated in processes of GI inflammation [24]. MMPs are proposed to be major factors for intestinal tissue injury, suggesting an important role for these enzymes in the process of tissue remodelling and destruction during inflammation [25]. MMPs are thought to play a major role in the pathophysiologic process of IBD [26]. MMP-2 and MMP-9 seem to have contrasting roles in the development of colitis despite

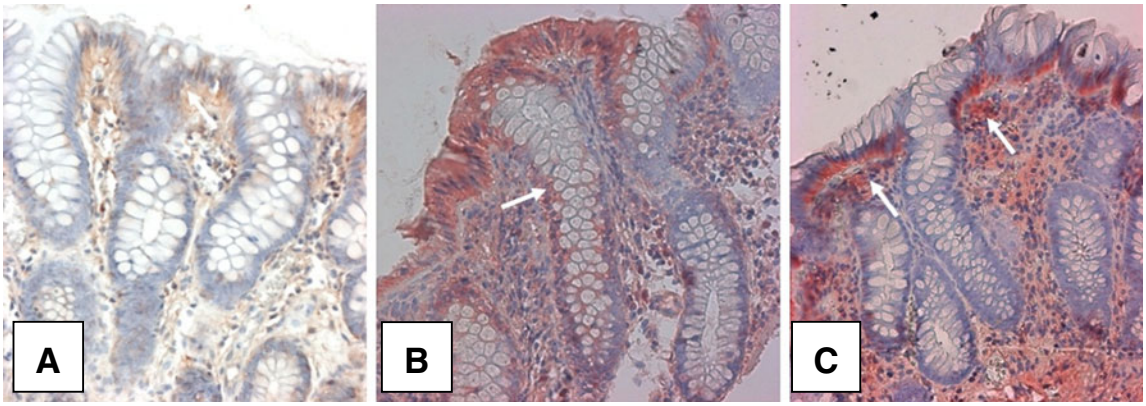

Fig. 1 Immunohostochemical expression of MMP-9 in normal colonic tissue and in ulcerative colitis. (**a**: normal healthy mucosa; **b**: mild inflammation; **c**: severe inflammation). (MMP-9-biotin-streptavidin-amino-aethyl-carbasole; hematoxylin eosin). **a** Normal colonic epithelium shows MMP-9 positive staining mainly within the cytoplasm and cytoplasmic membranes of the luminal colonic epithelial cells. Diffuse basal immunoreaction in almost every crypt, weak expression in lamina propria (*white arrow*) (300× magnification). **b** Stronger diffuse basal and cytoplasmic immunostaining of MMP-9 in epithelial cells of the crypts, weak MMP-9 expression of lymphoid aggregates in lamina propria (*white arrow*). (200× magnification). **c** Severe inflammation showing almost no expression of MMP-9 in crypts. A pronounced positive staining in lamina propria for MMP-9 in inflammatory cells was found (*white arrows*) (200× magnification)

structural similarities and up-regulation of both during colitis. MMP-9 is an important mediator of tissue injury in colitis, whereas MMP-2 protects against tissue damage and maintains gut barrier function. That MMP-9 drives colitis makes it a logical potential therapeutic target for IBD treatment [27]. The exact mechanism of the pathological process in MC is unknown. MC represents a self-limited disease entity arising from heterogeneous causative factors including stress or diet [28].

MMP-9 expression has not been determined in correlation with the severity of inflammation in UC and there are no data available in terms of MMP-9 expression in MC.

In the present study we evaluated the alteration of MMP-9 immunohistochemical expression in normal colonic mucosa, MC and mildly, moderately and severely inflamed colonic biopsy specimens from patients with UC. For additional (positive) controls MMP-9 immunohistochemical expression was also evaluated in inflamed mucosa from patients with active diverticulitis and in biopsies from patients with inactive diverticular disease. Immunostaining was determined semiquantitatively.

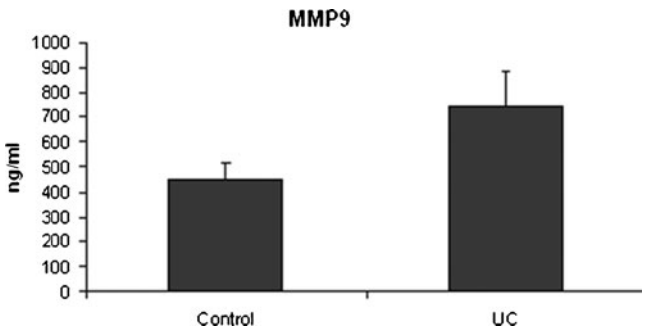

Fig. 2 Serum MMP-9 antigen concentrations in patients with ulcerative colitis ($n=5$) and controls ($n=7$) expressed in ng/ml (mean values and standard deviations)

We found that immunohistochemical expression of MMP-9 in MC was similar as compared to controls, or active diverticulitis and inactive diverticular disease. There was also no significant difference between LC and CC. In UC MMP-9 mucosal expression was significantly higher compared to MC, active diverticulitis, inactive diverticular disease or normal colonic mucosa. Because of the self-limiting nature of inflammation, it is little surprise, that no significant differences were observed regarding MMP-9 mucosal expression between LC and CC compared to active or inactive diverticular disease or controls.

Evaluation of MMP-9 expression in the different severity stages of UC showed that immunostaining was most pronounced in severe inflammation. MMP-9 expression was significantly higher in each inflammatory group of UC compared to controls.

Our gene expression microarray data and RT-PCR results correlate with the immunohistochemical behavior of MMP-9 in the colonic mucosa, showing a significantly higher expression of MMP-9 in severely active UC compared to healthy controls. Our data are in agreement with results by Gao et al. [29]. They reported a markedly increased MMP-9 protein and mRNA in inflamed mucosa of UC, with the highest levels in severely inflamed tissues, demonstrated by enzyme-linked immunosorbent assay (ELISA), zymography, activity assay and reverse transcription polymerase chain reaction (RT-PCR). This increased expression of MMP-9 and other MMPs was previously demonstrated by several other

groups by zymographic analysis, ELISA or PCR [15, 19, 30]. Our results with others suggest that the alteration of MMP-9 expression could contribute to the severity of mucosal damage in severely active UC. The preliminary ELISA results from sera of patients with UC also confirm our immunohistochemical data: serum MMP-9 antigen concentrations were significantly higher in patients with UC than those found in controls.

Our findings are relevant from both, biological and clinical point of view. It would be useful to have additional biomarkers to help clinicians better determine the risk of severe UC development. Although our results indirectly suggest that MMP-9 expression/activity can be a marker of mucosal damage and correlate to the severity of inflammation, MMP-9 does not seem to be a specific marker of IBD. However, this topic is open to debate: there is conflicting evidence in the potential use of MMPs in the differential diagnosis of IBD. Indeed, urinary MMP levels were suggested to be a good biomarker of IBD activity: MMP-2 and MMP-9 levels were independent predictors of both CD and UC. Sensitivity of MMP-s was established in IBD, but specificity in relation to various different colitis conditions was not demonstrated [31].

It can be speculated that relevance of MMP-9 in UC may also support a possible therapeutic approach. Indeed, experimental data suggest that inhibition of MMP-9 may be of benefit in future strategies in IBD therapy [32, 33]. In the context of the involvement of tumor necrosis factor-alpha (TNF-α) in the regulation of MMP-9 synthesis, it has been recently suggested that infliximab therapy can induce a down-regulation of MMP-9, which may also contribute to the therapeutic efficacy of this drug in IBD [34]. It has been also proposed that anti-TNF therapy may enhance the inhibitor TIMP-1 production and myofibroblast migration, which may reduce MMP activity and facilitate the wound healing [35]. Furthermore, specific inhibition of MMPs or compounds that can restore the balance between MMPs and their endogenous inhibitors should provide novel pharmacologic perspective against IBD [36, 37].

Our results demonstrate that mucosal up-regulation of MMP-9 expression in UC correlates with the severity of inflammation, whereas decreased expression of MMP-9 were seen in MC. These findings suggest relevant differences in the pathophysiologic mechanism between MC and UC. Decreased levels of interstitial MMPs and increased expression of TIMPs have been found in patients with MC suggesting that reduced matrix degradation and not over-activation of matrix synthesis leads to subepithelial accumulation of matrix proteins [38, 39]. On the other hand, the role of TNF-α in MC is not clear. A different cytokine profile including a lesser increase of TNF-α expression would explain the differences between MC and UC regarding severity and localization of mucosal alterations and the behavior of MMP-9 expression.

Our results indirectly suggest that MMP-9 could contribute to the severity of mucosal damage in severely active UC. Taken together, MMP-9 seems to be actively involved in the inflammatory and remodelling processes in UC. Together with other biological markers, quantification of MMP-9 might be useful to identify patients at higher risk for progression to severe UC. Furthermore, MMP-9 could be a target molecule for drug development: strategies to inhibit MMP-9 may be of potential therapeutic benefit in IBD.

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