Chapter 6

Expression of matrix metalloproteinases (MMP)-2 and MMP-9 in intestinal tissue of patients with inflammatory bowel diseases (IBD)

Short title: MMPs in IBD

Qiang Gao
Martin J.W. Meijer
Frank J.G.M. Kubben
Cornelis F.M. Sier
Laurens Kruidenier
Wim van Duijn
Marlies van den Berg
Ruud A. van Hogezand
Cornelis B.H.W. Lamers
Hein W. Verspaget

Department of Gastroenterology and Hepatology
Leiden University Medical Center
The Netherlands
Abstract

**Aim:** Assessment of the expression of matrix metalloproteinases (MMP)-2 and MMP-9 and their mRNA in intestinal tissue of patients with inflammatory bowel disease (IBD), in relation to the presence of inflammation.

**Methods:** Frozen resected tissue specimens from patients with Crohn's disease (CD) or ulcerative colitis (UC) and control tissue from patients with a colorectal carcinoma were used for enzyme-linked immunosorbent assay (ELISA), zymography and reverse transcription polymerase chain reaction (RT-PCR) evaluation of MMP-2 and MMP-9. Paraffin sections of resection specimens from patients of these groups were stained for these MMPs using indirect immunohistochemistry with polyclonal antibodies.

**Results:** The amount of MMP-2 as measured by ELISA showed an increase related to severity of inflammation in IBD tissues and compared to control tissue. In non-inflamed tissue, the level of MMP-2 was about 2-fold higher than in control tissue and in inflamed tissue this increase reached an almost 3-fold level, in both CD and UC. With zymography and RT-PCR a similar tendency of enhanced levels was observed in the IBD tissues. The immunohistochemical results showed that MMP-2 was prominently present in the extracellular matrix (ECM) of the submucosa with a lower but more generalized expression in severely inflamed regions, and somewhat stronger in UC than in CD.

The MMP-9 level was found to be elevated almost 4-fold in non-inflamed tissue, with a further increase up to 7-fold in inflamed CD and UC tissue, in comparison with control tissue, as determined by ELISA and zymography. Similar to the MMP-9 protein, the expression of mRNA was also increased in IBD tissues, and in relation to the severity of inflammation. The immunoreactivity to MMP-9 was most prominent as a positive granular staining in polymorphonuclear leukocytes (PMNL) and found to be increased in all IBD tissues. In addition, an increased MMP-9 expression in the ECM was observed in relation to the severity of inflammation.

**Conclusions:** MMP-2 and MMP-9 are enhanced in the intestinal tissue and seem to be actively involved in the inflammatory and remodeling processes in IBD, without major differences between CD and UC. Further evaluation of these factors in relation to treatment response and clinical course of the diseases is indicated.
Inflammatory bowel diseases (IBD), i.e., Crohn's disease (CD) and ulcerative colitis (UC), are characterized by inflammation and ulceration of the gastrointestinal tract, with numerous gastrointestinal tract and systemic complications, but of unknown specific etiology [1]. In the pathophysiological process of IBD, a variety of inflammatory mediators, such as proteolytic enzymes, cytokines and growth factors, and many kinds of cells, like leukocytes and stromal cells, are implicated in the tissue injury and healing processes.

Matrix metalloproteinases (MMPs) are a family of Zn$^{2+}$-containing neutral proteinases and thought to be major contributors to breakdown and reconstitution of extracellular matrix (ECM) in physiological processes, like tissue remodeling during development, growth and wound repair, and in pathological conditions, including destructive diseases, such as arthritis, atherosclerotic plaque rupture, and tumor progression [2-4]. In general, MMPs are secreted as inactive proenzymes that require proteolytic cleavage for activation. The proteolytic activity of MMPs is precisely regulated by the balance between zymogen activation and enzyme inhibition through endogenous inhibitors, such as $\alpha$-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs) [5]. To date over twenty members of the MMPs family have been found in vertebrates. Depending on substrate specificity, amino acid similarity, and identifiable sequence modules MMPs are divided into four major subgroups: collagenases, stromelysins, gelatinases, and membrane-type MMPs. Gelatinases are composed of two members: MMP-2 (gelatinase A), a 72 kDa proteinase, and MMP-9 (gelatinase B), a 92 kDa proteinase, which specifically degrade basement membrane (BM) type IV collagen, as well as gelatin, collagen type I, V, VII, X, elastin, laminin and fibronectin [6;7]. MMP-2, a most commonly expressed enzyme in normal adult tissue, is primarily produced by stromal cells. MMP-9 is mainly synthesized by inflammatory cells, particularly polymorphonuclear leukocytes (PMNL) [2;8-10]. The expression of MMP-2 and MMP-9 has been found to change in different situations, such as embryonic development, diverse pathophysiological conditions and during culture [5;11]. For example, MMPs are proposed to be major factors for intestinal tissue injury mediated by T cells in IBD. [12;13]. Previous studies from our and other groups showed an aberrant expression of MMPs in CD or UC, either on the protein or mRNA level, or immunohistochemically [14-21]. In the present study, we evaluated the expression of MMP-2 and MMP-9 protein, both in level and localization, and their mRNA gene products in relation to mucosal inflammation in patients with CD or UC.

**Materials and Methods**

**Tissue samples**

The samples in this study were obtained from surgical resection specimens and include pairs of macroscopically inflamed and normal appearing (non-inflamed) mucosa from patients with CD or UC, both clinically and histologically confirmed, with normal tissue from patients with a colorectal carcinoma, at least 10 cm from the tumor, as controls. Details on the tissue specimens included and patients characteristics are described in a previous study [22]. For the enzyme-linked immunosorbent assay (ELISA) assessments frozen tissues were used from 16 patients with CD, 14 patients with UC, and from 16 controls. The reverse transcription polymerase chain reaction (RT-PCR) samples consisted of 5 CD, 5 UC and 10 controls. A total of 47 surgically resected, formalin-fixed, paraffin-embedded intestinal tissue specimens, obtained from the Pathology Department of the Leiden University Medical Center, were immunohistochemically stained for MMP-2 (CD: n=15, UC: n=14 and control: n=15) and for MMP-9 (CD and UC: n=12, and control: n=16), respectively.
Determination of MMPs by ELISA and zymography

Frozen intestinal tissue samples were homogenized on ice by adding 1 ml 0.1 M Tris-HCl/0.1% Tween-80 buffer per 60 mg sample [22]. MMP-2 and MMP-9 levels in the homogenized tissue samples were measured by our highly specific enzyme-linked immunosorbent assays (ELISA), which measure the grand total of pro-enzyme, active- and inhibitor-complexed forms of the respective MMP, as described previously [23;24]. In brief, a polyclonal anti-MMP-2 antibody or monoclonal anti-MMP-9 antibody was used as catching antibody and appropriately diluted samples were incubated overnight at 4 °C. Immunodetection of MMP-2 was performed using polyclonal anti-MMP-2 followed by biotin-labelled goat anti-rabbit-IgG and of MMP-9 with biotin-labelled polyclonal anti-MMP-9 antibody. After incubation with avidin-peroxidase the chromogenic substrate 3,3’, 5,5’-tetramethyl benzidine in the presence of hydrogen peroxide was added and the reaction was stopped with H2SO4 and the absorption was measured at 450 nm. The amount of MMP was calculated from the parallel standard curve and expressed in ng MMP per mg protein.

Gelatin-zymography was performed to determine the level of the active and pro-enzyme forms of MMPs, as described previously [25]. In brief, standardized homogenate protein amounts were electrophorized on gelatin-polyacrylamide gels, which were subsequently stained and analysed for gelatin digestion by laser densitometry. The levels were expressed in arbitrary units (AU) related to a uniform internal standard used in each gel.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Oligonucleotide primers (Table 1) for the RT-PCR were derived from the DNA sequence database of the NCBI (Bethesda, MD, USA). Primer sets were designed using the Primers3 Output computer program (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The MMP-2 and MMP-9 PCR products span three introns to prevent interference of contamination by genomic DNA. Specificity of the primers was checked with the NCBI BLAST program.

β-actin was used as a control to normalize PCR signals from the different samples.

Total RNA was isolated from tissue samples by the method of Chomczynski and Sacchi [26]. The integrity and quality of the purified RNA were analyzed by 1.5% agarose gel/ethidium-bromide staining and the 260/280 nm absorbance ratio. Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase was used for cDNA synthesis from DNAs treated RNA. The PCR was started at 94°C for 3 min. followed by 30 cycles for MMP-2 and MMP-9, and 28 cycles for β-actin. Each cycle consisted of a denaturation step (at 94°C for 30 sec.), an annealing step for 45 sec. (at 56°C for MMP-2 and β-actin, 59°C for MMP-9) and extension step (at 56°C for MMP-2 and β-actin, 59°C for MMP-9) and extension step (at 72°C for 1 min.), followed by a final elongation step (at 72°C for 7 min.). The reaction was performed in a Whatman T Gradient cycler (Biometra, Goettingen, Germany) and the amplified products were electrophorized on 1.5% agarose gels containing ethidium-bromide (0.5µg/ml) and visualized under ultraviolet light. A RT-PCR, which contained RNA but not M-MLV reverse transcriptase, was used to check contamination with genomic DNA. The Scion imaging program (Frederick, Maryland, USA. www.scioncorp.com) was used to semi-quantify the band density in the gels, expressed in arbitrary units (AU).

| Table 1. Oligonucleotide primers for RT-PCR |
|-----------------|-----------------|-----------------|------------------|
| mRNA | Gene | Sense primer | Antisense primer | Product size |
| NM-004530 | MMP-2 | AGGATCATTTGGCTACACACC | AGCTGTCATAGGATGTGCCC | 535 |
| NM-004994 | MMP-9 | CGCAGACATCGTCATCCAGT | GGATTGGCCTTGGAAGATGA | 406 |
| NM-001101 | β-actin | GGTCAGAAGGATTCTTATG | GGCTCAAAACATGATCTG | 238 |
Immunohistochemical staining for MMPs

To assess the localization of MMP-2 and MMP-9 within the intestinal tissues, indirect immunohistochemical staining of the MMPs was performed as described previously [24]. In brief, paraffin tissue sections, treated with proteinase K for MMP-2 antigen retrieval, were incubated with rabbit polyclonal anti-human MMP antibodies, similar to those used in the ELISAs. Subsequently, the sections were incubated with biotinylated goat anti-rabbit Ig, peroxidase-labelled streptavidin, and stained with 3-amino-9-ethylcarbazole and hematoxylin. The immunohistochemical staining was semiquantitatively assessed using the following scoring system: 0 = no staining, 1 = a few positive cells / areas of tissue or a low staining intensity in all cells, 2 = a minority of the cells / areas of tissue positive or a moderate staining intensity in all cells, 3 = a majority of the cells / areas of tissue positive and/or a moderate staining intensity in all cells, 4 = all cells or areas of tissue strongly positive.

Statistical analysis

The ELISA, zymography and RT-PCR results are given as mean ± S.E.M. and those of the immunohistochemical evaluation as median with inter quartile range. Significance of the differences between groups were assessed using the Mann-Whitney U-test or the Wilcoxon signed-ranks test for paired data. Differences were considered significant when $P \leq 0.05$.

Results

MMP-2

The amount of MMP-2 in the intestinal tissue, as determined by ELISA, showed a tendency to increase in relation with severity of inflammation in IBD (Figure 1). In non-inflamed IBD tissue, the amount of MMP-2 was elevated to a near 2-fold higher level compared to control tissue [11.6±1.1 vs. 6.9±0.8 (ng/mg protein)]. In inflamed IBD tissue the MMP-2 level was even higher (16.7±1.7), though not significantly different from non-inflamed tissue. The MMP-2 levels between similar tissues of CD and UC didn’t show any statistically significant difference, in both inflamed and non-inflamed tissues of the diseases, but all were significantly higher than in the control tissues (Table 2). The zymographic analyses revealed that both the active and pro-enzyme form of MMP-2 is increased in IBD, without major differences between inflamed and non-inflamed tissues (Figure 2) and between CD and UC (data not shown). With RT-PCR also a 2- to 3-fold increase in the expression of MMP-2 mRNA was found in the IBD tissues, although less impressive compared to the protein levels, in comparison to control tissues (Figure 3).

| Table 2. ELISA results of MMP-2 and MMP-9 in intestinal tissues |
|-----------------|-----------------|-----------------|
|                 | Control (n=16)  | Crohn’s disease (n=16) | Ulcerative colitis (n=14) |
|                 | Non-inflamed    | Inflamed         | Non-inflamed    | Inflamed |
| MMP-2           | 6.9 ± 0.8       | 9.9 ± 1.3*       | 14.4 ± 2.0*     | 13.4 ± 1.8*       | 19.2 ± 2.9*    |
| MMP-9           | 6.0 ± 1.0       | 27.7 ± 6.3*      | 38.2 ± 8.7* (7) | 23.3 ± 9.3*       | 51.1 ± 10.2* (7) |

MMPs presented in ng/mg protein (mean ± S.E.M.)

*: $0.0005 < P < 0.04$ vs controls, (7): $P = 0.08$; †: $P = 0.02$ vs non-inflamed.
Immunohistochemically MMP-2 was most prominently present in the ECM of the submucosa in every tissue group (Table 3 and Figure 4 A). This diffuse MMP-2 staining was patchy and relatively strong in the deeper layer of the submucosa in the control tissues and in non-inflamed regions of IBD tissues, but was found to be significantly decreased in severely inflamed regions, where it was seen throughout the submucosa (Figure 4 B). This decreased staining intensity in inflamed tissue was observed in CD as well as in UC, although in the latter the overall MMP-2 staining was found to be more prominent [non-inflamed: UC 2.5 (1.3-2.5) vs CD 1.3 (0.5-2.0), and inflamed: UC 1.5 (1.0-1.5) vs 0.5 (0.5-1.0); both P=0.02]. In addition, a weak diffuse cytoplasmic staining for MMP-2 was present in some epithelial cells of all tissue groups. In some cases we also found a positive staining of the basal membrane (BM) underneath the luminal epithelium, not related to the positive staining in epithelial cells (data not shown).

MMP-9

MMP-9 in the intestinal mucosa was found to have a similar pattern to that of MMP-2. An elevated protein level in non-inflamed IBD tissue, nearly 4-fold increased (25.7±5.4 vs. 6.0±1.0), and further increased in inflamed IBD tissue, up to 7-fold (44.3±6.7), compared to controls (Figure 1). Again no significant differences in the MMP-9 levels between inflamed tissues of CD and UC, as well as between non-inflamed tissues, were found (Table 2), but all were enhanced compared to control tissues. With zymography both the active and pro-enzyme form of MMP-9 was found to be significantly increased in IBD, but only pro-MMP-9 was found to increase further with inflammation (Figure 2). In similarity to the protein level, the expression of MMP-9 mRNA was also increased in the IBD tissues, 2-fold in non-inflamed about 4-fold in inflamed tissue, although no statistical significance was reached (Figure 3).

Table 3. Predominant immunohistochemical expression of MMP-2 and MMP-9 in intestinal tissues

<table>
<thead>
<tr>
<th></th>
<th>Control (n=16)</th>
<th>Inflammatory bowel disease</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Non-inflamed</td>
<td>Inflamed</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Submucosa</td>
<td>2.0 (1.5-2.5)*</td>
<td>2.0 (1.0-2.5)</td>
<td>1.0 (0.5-1.5)*†</td>
<td>(n=29)</td>
</tr>
<tr>
<td>MMP-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMNL</td>
<td>0.8 (0.5-1.9)</td>
<td>2.0 (1.0-2.0) *</td>
<td>2.0 (1.5-2.9) *</td>
<td>(n=24)</td>
</tr>
<tr>
<td>Matrix</td>
<td>0.5 (0.5-0.5)</td>
<td>0.5 (0-0.5)</td>
<td>1.0 (0.5-1.5) *†</td>
<td>(n=24)</td>
</tr>
</tbody>
</table>

PMNL: polymorphonuclear leukocytes; *: in median (inter quartile range), †: P ≤ 0.0005 vs controls, ††: P ≤ 0.002 vs non-inflamed.
Immunohistochemically, a pronounced positive staining for MMP-9 in PMNL was found throughout the intestinal walls. An increased immunoreactivity of granular MMP-9 positive PMNL was observed in all IBD tissues, both non-inflamed and inflamed (Figure 5 A, B and Table 3). In addition, a weak positive reaction of MMP-9 was found in the ECM, which was found to be increased with severity of the inflammation in IBD tissues (Table 4). Occasionally macrophages positive for MMP-9 were found, predominantly in inflamed areas (Figure 5 D and E). In normal tissues and non-inflamed tissues IBD no other major cell types showed MMP-9 staining. Overall, no differences in the expression of MMP-9 between CD and UC were found (data not shown).

![MMP level graph](image1)

**Figure 2.** MMP-2 (hatched bars) and MMP-9 (dotted bars) zymography results in mean ± S.E.M. Both the active and pro-enzyme forms of MMP-2 and MMP-9 were increased in IBD tissues, only pro-MMP-9 increased further with inflammation.

* P ≤ 0.02 vs controls, † P = 0.0005 vs non-inflamed,

AU: arbitrary units, IBD: inflammatory bowel disease, act: active enzyme, pro: pro-enzyme.

![mRNA level graph](image2)

**Figure 3.** MMP-2 (open bars) and MMP-9 (hatched bars) RT-PCR results in mean ± S.E.M. Similar to the expression pattern of the proteins there was an increase in mRNA in IBD tissues, and in relation with inflammation, compared to that in control tissues.

* P = 0.09; † P = 0.03 vs controls,

AU: arbitrary units, IBD: inflammatory bowel disease.
Inflammatory bowel disease is characterized by a high intestinal tissue turnover during the sequence of inflammation, tissue destruction and healing. During inflammatory processes there are different phases, which include the acute reaction, breakdown and proliferation of cells, and remodeling of tissue, with overlap between these phases, indicating that destruction and healing of tissue form a continuum. In the present study we found the expression of MMP-2 at both the protein and mRNA level to be increased in intestinal IBD tissues, with the highest levels in inflamed areas. Immunohistochemical evaluations showed that in the severely inflamed regions the MMP-2 staining was distributed throughout the ECM within the mucosa and submucosa, although the intensity was less than in controls and non-inflamed IBD tissues, where it was found only in the submucosa. Apparently, there is a generalized demand of MMP-2 from inflammatory tissues in IBD, indicative of MMP-2 involvement in the enhanced intestinal tissue turnover.

MMP-2 is most commonly expressed and can be isolated in large quantities from normal quiescent tissues and is believed to participate in maintenance of collagen homeostasis within tissues [27-29]. Baugh et al. [17] postulated that the activation of MMP-2 contributed to the degradation of the basal membrane type IV collagen and loss of epithelial organization in active IBD. More recent studies also showed the enhanced expression of MMP-2 in IBD intestine, particularly in the stromal compartment, e.g. (myo)fibroblasts, which participate in the ECM remodeling, collagen and basement membrane turnover leading to intestinal ulceration, epithelial damage and/or fistula formation [18-20;30]. From these studies and our observations we conclude that MMP-2 mainly participates in the intestinal tissue remodeling, in addition to its role in acute inflammation in IBD. MMP-2 is known to be able to cleave/activate cytokines, growth factors and MMPs themselves [31-33]. The resultants, processed by MMP-2, are important modulators in the inflammatory reactions and healing process in IBD, such as interleukin (IL)-1β, an important proinflammatory cytokine, fibroblast growth factor receptor-1 ectodomain, which modulates the angiogenic activity of FGF, inactivated monocyte chemoattractant protein (MCP)-3, all affecting the inflammatory response [34;35].

There seems to be consensus about the increased protein level of MMP-2 in intestinal IBD tissues, either determined by ELISA, zymography or western blotting [17;19;21]. Yet, about the localization and mRNA expression of MMP-2 in IBD there remains some controversy. The predominant cell type identified to contain MMP-2 mRNA were the myofibroblasts underneath the epithelium and fibroblasts within the submucosa [18;21;30]. Immunohistochemically, however, either no [15], diffuse (present study), or diverse positive cells [21] were seen. One of the main reasons of this inconsistency is probably sequestration of MMP-2 to the matrix components which prohibits an exact localization [36]. Similarly to Van Lampe et al. [18], we found a (marginally) elevated MMP-2 mRNA level in inflamed intestinal IBD tissue. Although the post-transcriptional regulation of MMP-2 is probably more important than the transcriptional regulation in the expression of MMP-2, the enhanced MMP-2 mRNA expression in IBD might also be the result of a higher demand due to tissue remodeling rather than the effect of inflammatory mediators on the transcription of the MMP-2 gene. The regulation of the MMP-2 gene transcription, for example, is not induced by proinflammatory cytokines, like IL-1 or tumor necrosis factor (TNF)-α, because the promoter region of MMP-2 lacks an AP-1 binding site, as present in other MMP genes, which binds the transcriptional factor AP-1 induced by proinflammatory agents [9]. Yet, transforming growth factor (TGF)-β, which is a potential modulator and inhibitor of the inflammatory reaction in IBD, induces the transcription and prolongs the half-life time of MMP-2 mRNA [9;37]. Thus, the production of MMP-2 seems to be delicately regulated during inflammatory and healing processes in IBD.

Discussion
In general, the constitutive expression of MMPs is rather low, with the exception of the neutrophil MMPs MMP-8 and MMP-9 [3;4]. It is believed that MMP-9 is stored in the secondary and tertiary granules of neutrophils for rapid release into inflammatory sites [4]. We found the amount of MMP-9 protein to be increased in relation to inflammation in IBD tissue, in both CD and UC. This increased expression of MMP-9 was previously reported by several other groups, either by zymographic analysis or immunohistochemically, confirmed by in situ hybridization for mRNA [14;17;21]. Uniformly the PMNL were identified as the main source of MMP-9 within intestinal tissues, with occasionally positive macrophages, whereas myofibroblasts were found to be MMP-9 negative, the latter also confirmed by in vitro studies [30]. A diffuse matrix/ECM positivity for MMP-9 was also occasionally noticed, probably caused by sequestration of the excreted MMP-9 to the stroma, as mentioned previously for MMP-2. Interestingly, all studies reported an enhanced level of MMP-9 in the non-involved/non-inflamed IBD tissue, sometimes without a clear differences with inflamed tissue [17]. This phenomenon seems to be due to the fact that macroscopically non-involved IBD tissue does have an increased amount of inflammatory cells, particularly PMNL containing MMP-9 [17], as exemplified by increased myeloperoxidase levels [38].

In general the studies demonstrate that MMP-9 participates actively in the inflammatory process of IBD, especially in the acute phase. An improper activity of MMP-9 is thought to cause the destruction of tissue via the degradation of components of the ECM and, in addition, to influence the generation or activation of neutrophilic chemokines, promoting neutrophil migration across the basement membrane of capillaries [2;4;7;39]. The ECM fragments produced by the activation of MMP-9 are also known to be chemotactic for inflammatory cells [6]. In a previous study we also showed that TNF-α is an important regulator of the transcription of MMP-9 mRNA in blood leukocytes from patients with CD [40]. Furthermore, Sanceau et al. [41] reported that TNF-α can activate the MMP-9 gene transcription through regulation of the nuclear factor (NF)-κB activation. Recently, Kirkegaard et al. [21] reported that MMP-9 contributes to fistula formation in CD through degradation of the ECM, whereas, on the other hand, it has been reported that MMP-9 has a function in tissue repair by facilitating re-epithelialization and degradation of denatured collagen [29]. Thus, similarly to MMP-2 also MMP-9 seems to contribute to the different phases of the intestinal inflammatory process, from damage to repair. In the context of the involvement of TNF-α in the regulation of MMP-9 synthesis it would be interesting to assess the effects of the very effective treatment of patients with Crohn's disease with the (chimeric monoclonal) antibodies against TNF-α on the expression of this MMP.

Taken together, MMP-2 and MMP-9 in the intestinal tissue seem to be actively involved in the inflammatory and remodelling processes in IBD. Further evaluation of these factors in relation to treatment response and clinical course of the diseases is warranted.

References


