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Matrix metalloproteinases in inflammatory bowel disease : expression, regulation and clinical relevance

Meijer, M.J.W.

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Chapter 2

Expression of Matrix Metalloproteinases-2 and -9 in Intestinal Tissue of Patients with Inflammatory Bowel Disease (IBD)

Short title: gelatinases 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

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Abstract

Background/aims. Matrix metalloproteinases are major contributors in the breakdown and reconstitution of basement membranes and extracellular matrix in pathophysiological processes. We assessed the expression of matrix metalloproteinases-2 and -9 in intestinal tissue of patients with inflammatory bowel disease.

Patients/methods. Resected tissue specimens from patients with Crohn's disease or ulcerative colitis and control tissue from patients with a colorectal carcinoma were used for enzyme-linked immunosorbent assay, zymography, activity assay, reverse transcription polymerase chain reaction and immunohistochemistry to evaluate the expression of these matrix metalloproteinases.

Results. Matrix metalloproteinase-2 and more strongly matrix metalloproteinase-9 protein and mRNA were markedly increased in inflammatory bowel disease tissues, with the highest levels in severely inflamed tissues. Immunohistochemistry showed that matrix metalloproteinase-2 was present in the extracellular matrix of the submucosa, with a lower but more generalized expression in the severely inflamed regions. Matrix metalloproteinase-9 was most prominent in polymorphonuclear leukocytes and was increased, also in activity, in all inflammatory bowel disease tissues. An increased matrix metalloproteinase-9 expression in the extracellular matrix was observed in relation to the severity of inflammation.

Conclusions. Matrix metalloproteinases-2 and -9 are enhanced in the intestinal tissue and seem to be actively involved in the inflammatory and remodelling processes in inflammatory bowel disease, without major differences between CD and UC.

Introduction

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 aetiology.[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, leukocytes and stromal cells, are implicated in the tissue injury and healing processes.[2] Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-containing neutral proteinases and are thought to be major contributors to breakdown and reconstitution of extracellular matrix (ECM) in physiological processes, like tissue remodelling during development, growth and wound repair, and in pathological conditions, including destructive diseases, such as arthritis, atherosclerotic plaque rupture and tumour progression.[3-5] 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 α-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs).[6] To date, over 20 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 kD 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.[7,8] 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).[3,9-11] 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.[6,12] For example, MMPs are proposed to be major factors for intestinal tissue injury mediated by T cells in IBD.[13,14] Previous studies by other groups and us showed an aberrant expression of MMPs in CD or UC, either on the protein or

mRNA level, or immunohistochemically.[15-22] 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.

Patients and methods

Patients and 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 tumour, as controls. Details on the tissue specimens included and patients characteristics are described in a previous study.[23] For the enzyme-linked immunosorbent assay (ELISA) assessments, frozen tissues were used from 16 patients with CD, 14 patients with UC and 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, zymography and activity assays

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.[23] MMP-2 and MMP-9 levels in the homogenized tissue samples were measured by our highly specific ELISAs, which measured the grand total of pro-enzyme, active- and inhibitor-complexed forms of the respective MMP, as previously described.[24,25] 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 ^{0}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 H_2SO_4 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 previously described.[26] In brief, standardized homogenate protein amounts in 2% (w/v) sodium dodecyl sulphate were electrophorised on gelatin-polyacrylamide gels, through which the MMP isoforms are separated on molecular weight and the pro-enzymes become activated. The gels were subsequently incubated overnight at 37 ^{0}C , stained and analyzed for gelatin digestion by laser densitometry. The levels were expressed in arbitrary units (AU) related to a uniform internal standard, consisting of a colorectal cancer homogenate, containing MMP-2 and MMP-9, used in each gel. Active MMP-2 and MMP-9 were also determined by highly specific biochemical immunosorbent activity assays (BIA). In brief, microtitre plates were coated with the respective MMP antibodies as for the ELISAs. Samples at various appropriate dilutions were added and incubated overnight at 4 ^{0}C. MMP activity was determined by adding modified pro-urokinase and peptide substrate S-2444, with subsequent absorption measurement at 405 nm overtime and finally expressed in U/mg protein.[25]

Reverse transcription polymerase chain reaction

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.[27] The integrity and quality of the purified RNA were analysed 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 DNAse-treated RNA. The PCR was started at 94 ^{0}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 ^{0}C for 30 s.), an annealing step for 45 s (at 56 ^{0}C for MMP-2 and β-actin, 59 ^{0}C for MMP-9) and extension step (at 72 ^{0}C for 1 min), followed by a final elongation step (at 72 ^{0}C for 7 min). The reaction was performed in a Whatman T Gradient cycler (Biometra, Goettingen, Germany) and the amplified products were electrophorised on 1.5% agarose gels containing ethidium-bromide (0.5 g/ml) and visualized under ultraviolet light. An 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. The MMP/β-actin ratios times 10 for MMP-2 and times 100 for MMP-9 were expressed as AU.

Table 1. Oligonucleotide primers for RT-PCR

MRNA	Gene	Sense primer	Antisense primer	Product size
NM-004530	MMP-2	AGGATCATTGGCTACACACC	AGCTGTCATAGGATGTGCCC	535
NM-004994	MMP-9	CGCAGACATCGTCATCCAGT	GGATTGGCCTTGGAAGATGA	406
NM-001101	β-actin	GGGTCAGAAGGATTCCTATG	GGTCTCAAACATGATCTGGG	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.[23,25] 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 haematoxylin. Control sections incubated with pre-immune serum or buffer instead of the primary antibodies showed no staining. 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 and 4 = all cells or areas of tissue strongly positive.

Statistical analysis

The ELISA, zymography, BIA 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 (Fig. 1). In non-inflamed IBD tissue, the amount of MMP-2 was elevated to a near twofold higher level compared to control tissue [11.6 ± 1.1 ng/mg protein versus 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 did not 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 the pro-enzyme form of MMP-2 are increased in IBD (Fig. 2), without major differences between inflamed and non-inflamed tissues (Fig. 3) and between CD and UC (Table 3). Despite the increased protein levels, as determined by ELISA and zymography, the biochemical activity of MMP-2 was found to be not increased in both non-inflamed and inflamed CD and UC tissue (Table 4). With RT-PCR also, a two- to threefold increase in the expression of MMP-2 mRNA was found in the IBD tissues, although the increase was less impressive compared to the protein levels, in comparison to control tissues (Fig. 4). Immunohistochemically MMP-2 was most prominently present in the ECM of the

Table 2. ELISA results of MMP-2 and MMP-9 in intestinal tissues

	Control (n = 16)	CD (n = 16)		UC (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*,†	23.3 ± 9.3*	51.1± 10.2*,††

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

* 0.0005 < P < 0.04 vs. controls.

† P = 0.08 vs. non-inflamed.

†† P = 0.02 vs. non-inflamed.

Table 3. Zymography results of MMP-2 and MMP-9 in intestinal tissues

	Control (n=16)	CD (n = 14)		UC (n = 14)	
		Non-inflamed	Inflamed	Non-inflamed	Inflamed
MMP-2					
Act	0.29 ± 0.06	0.99 ± 0.30	0.69 ± 0.16 *	0.28 ± 0.06	0.99 ± 0.17 * ††
Pro	0.64 ± 0.10	4.11 ± 0.93 *	2.16 ± 0.42 *	1.96 ± 0.23 *	2.79 ± 0.40 * †
MMP-9					
Act	0.62 ± 0.13	4.41 ± 1.22 *	2.66 ± 0.54 *	1.78 ± 0.62	3.98 ± 0.61 * ††
Pro	1.18 ± 0.19	6.43 ± 0.94 *	8.32 ± 1.24 *	2.64 ± 0.48 *	9.41 ± 1.00 * ††

MMPs presented in AU (mean ± S.E.M.).

* 0.0005 < P < 0.05 vs. controls, † P = 0.08 vs. non-inflamed, †† P < 0.004 vs. non-inflamed

Table 4. BIA assay results of MMP-2 and MMP-9 in intestinal tissues

	Control (n=16)	CD (n = 14)		UC (n = 14)	
		Non-inflamed	Inflamed	Non-inflamed	Inflamed
MMP-2	8.1 ± 1.8	6.1 ± 2.0	6.3 ± 3.0	1.9 ± 1.0 *	8.0 ± 3.9
MMP-9	6.2 ± 2.5	11.4 ± 3.3	14.9 ± 4.8	32.2 ± 8.7 *	54.5 ± 14.4 * †

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

* 0.0005 < P < 0.05 vs. controls, † P = 0.008 vs. non-inflamed.

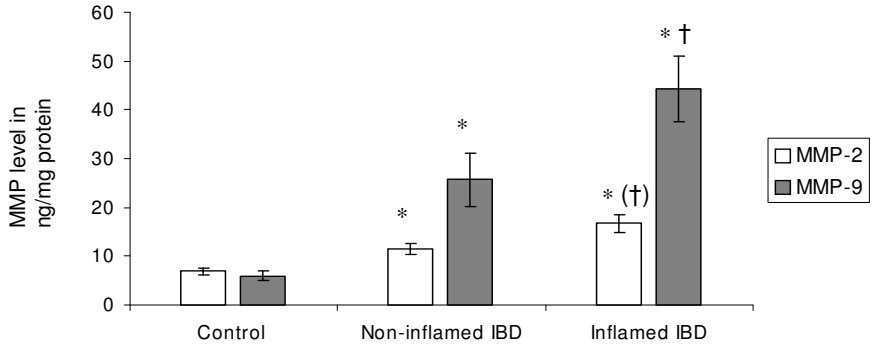

Figure 1. MMP-2 (open bars) and MMP-9 (grey bars) ELISA results in mean ± S.E.M. There was a highly significant difference between control and IBD tissues; the MMP levels increased with inflammation. $^{*}P \leq 0.005$ vs. controls;$^{(\dagger)}$ $P = 0.07$; $^{\dagger}P = 0.005$ vs. non-inflamed. IBD: inflammatory bowel disease.

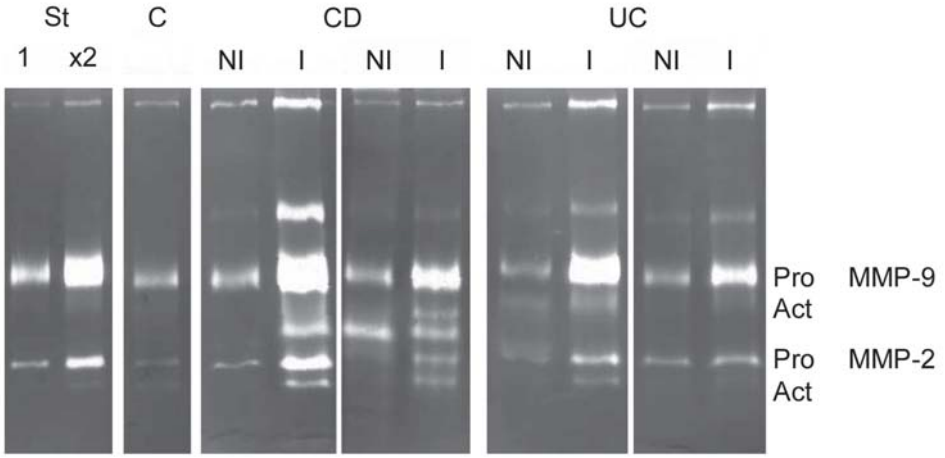

Figure 2. Example gelatin zymograms of non-inflamed and inflamed IBD tissue homogenates compared to control tissue and internal standard, identifying pro- and active MMP-2 and MMP-9. St: internal standard, C: control, CD: Crohn's disease, UC: ulcerative colitis, NI: non-inflamed, I: inflamed, Pro: pro-enzyme, Act: active enzyme

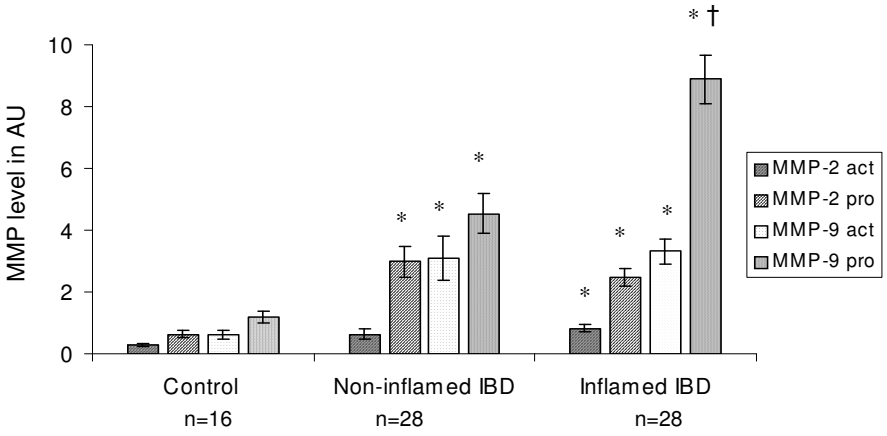

Figure 3. MMP-2 (hatched bars) and MMP-9 (dotted bars) zymography results in mean ± S.E.M. Both the active and pro-enzyme forms of MMP-2 and MMP-9 were increased in IBD tissues, only pro-MMP-9 increased further with inflammation. $^{*}P \leq 0.02$ vs. controls; †$P = 0.0005$ vs. non-inflamed, AU: arbitrary units, IBD: inflammatory bowel disease, act: active enzyme, pro: pro-enzyme

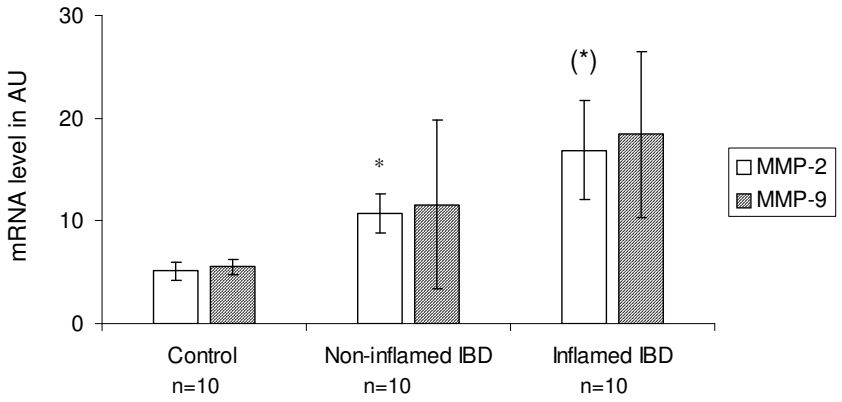

Figure 4. MMP-2 (open bars) and MMP-9 (hatched bars) RT-PCR results in mean ± S.E.M. Similar to the expression pattern of the proteins there was an increase in mRNA in IBD tissues, and in relation with inflammation, compared to that in control tissues. $^{(*)}P = 0.09$; $^{*}P = 0.03$ vs. controls. AU: arbitrary units, IBD: inflammatory bowel disease.

submucosa in every tissue group (Table 5 and Fig. 5A). 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 (Fig. 5B and C). 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) versus CD 1.3 (0.5–2.0) and inflamed: UC 1.5 (1.0–1.5) versus CD 0.5 (0.5–1.0); both P = 0.02]. In addition, a weak diffuse cytoplasmic staining for MMP-2 was

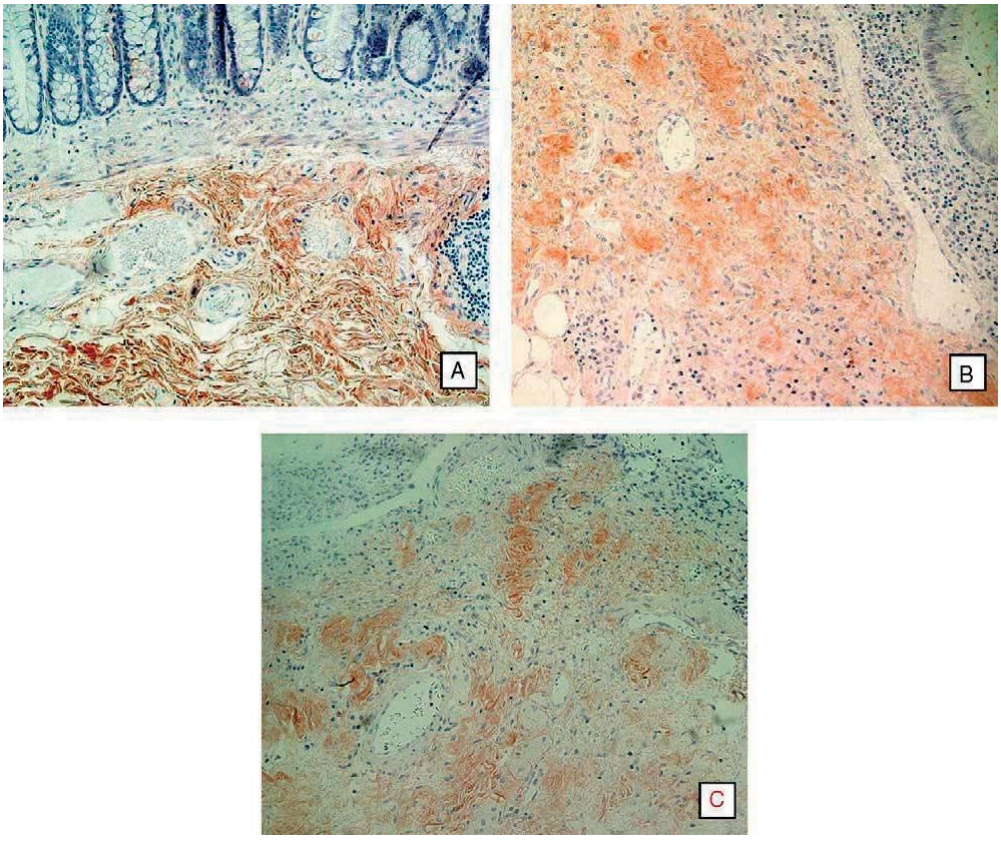

Figure 5. The prominent location of MMP-2 was in the submucosa, especially in controls (A, ×200); in inflamed tissues of CD and UC (B and C, ×200) MMP-2 was seen as a weak but more generalized positive reaction in the ECM.

present in some epithelial cells of all tissue groups. In some cases, we also found a positive staining of the basement membrane (BM) and/or (myo)fibroblasts underneath the luminal epithelium, not related to the positive staining in epithelial cells (data not shown).

Table 5. Predominant immunohistochemical expression of MMP-2 and MMP-9 in intestinal tissues

	Control (*n*=16)	IBD		
		Non-inflamed	Inflamed	
MMP-2				
submucosa	2.0 (1.5-2.5) [a]	2.0 (1.0-2.5)	1.0 (0.5-1.5) *†	(*n* = 29)
MMP-9				
PMNL	0.8 (0.5-1.9)	2.0 (1.0-2.0) *	2.0 (1.5-2.9) *	(*n* = 24)
Matrix	0.5 (0.5-0.5)	0.5 (0.0-0.5)	1.0 (0.5-1.5) *†	(*n* = 24)

PMNL: polymorphonuclear leukocytes

[a] In median (inter-quartile range); * $0.0005 \leq P \leq 0.02$ vs. controls, † $P \leq 0.002$ vs. non-inflamed.

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 increased nearly fourfold (25.7 ± 5.4 versus 6.0 ± 1.0), and it further increased in inflamed IBD tissue up to sevenfold (44.3 ± 6.7), compared to controls (Fig. 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 the pro-enzyme form of MMP-9 were found to be significantly increased in IBD (Fig.2), and predominantly pro-MMP-9 was found to increase further with inflammation (Fig. 3) in both CD and, particularly, UC (Table 3). In addition, the biochemical activity of MMP-9 was found to be increased in non-inflamed and inflamed CD and UC tissue, although it only reached statistical significance in UC (Table 4). Similar to the protein level, the expression of MMP-9 mRNA was also increased in the IBD tissues, twofold in non-inflamed and about fourfold in inflamed tissue, although no

statistical significance was reached (Fig. 4). 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, compared to controls (Fig. 6A-C and Table 5). 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 5). Occasionally macrophages positive for MMP-9 were found, predominantly in inflamed areas (Fig. 6D and E). In normal tissues and non-inflamed IBD tissues, 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).

Discussion

IBD is characterised 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 remodelling 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, as analyzed on full thickness mucosal tissues. 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. Yet, this was not reflected by an enhanced activity of MMP-2 as assessed by the BIA assay of the homogenates. Thus, the increased MMP-2 protein level does not seem to be under-balanced by endogenous inhibitors with respect to its activity. MMP-2 is most commonly expressed and can be isolated in large quantities from normal quiescent tissues and is believed to participate in the maintenance of

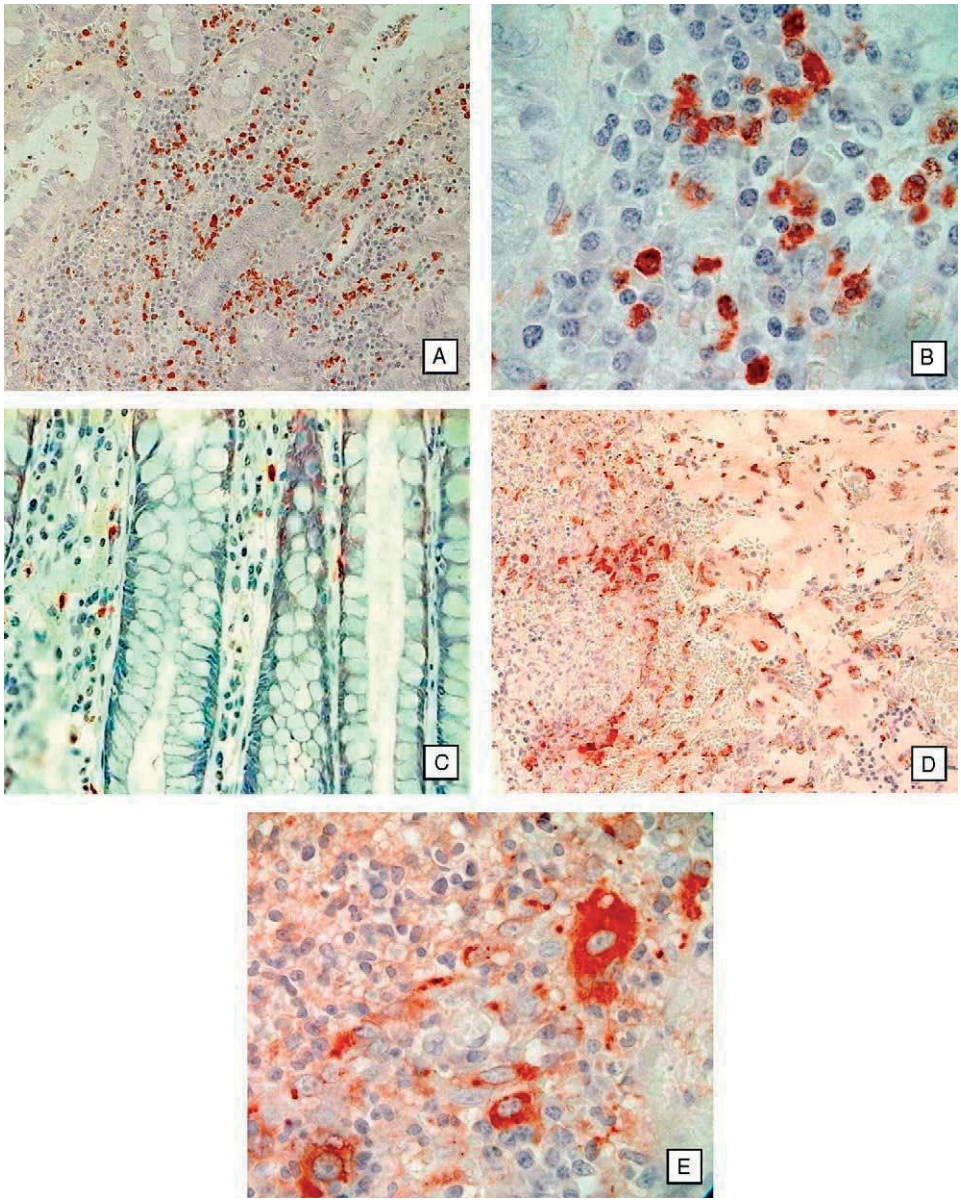

Figure 6. A pronounced positive staining for MMP-9 in leukocytes in inflamed areas of CD was found (A, ×200); neutrophils in inflamed tissue of CD were strongly positive for MMP-9 (B, ×1000); few leukocytes in control tissue were MMP-9 positive (C, ×400); MMP-9 in cells and ECM in UC inflamed area surrounding a necrotic region (D, ×200); and MMP-9 positive macrophages in inflamed tissue of UC (E, ×1000).

collagen homeostasis within tissues.[28-30] Baugh *et al.*[18] 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. In addition, Pender *et al.*[31] showed that membrane type-1 MMP, an important activator of MMP-2, is upregulated in IBD tissue. More recent studies also showed the enhanced expression of MMP-2 in IBD intestine, particularly in the stromal compartment, e.g. (myo)fibroblasts, which participates in the ECM remodelling, collagen and basement membrane turnover leading to intestinal ulceration, epithelial damage and/or fistula formation.[19-21,32] From these studies and our observations we conclude that MMP-2 mainly participates in the intestinal tissue remodelling, 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.[33-35] The resultants, processed by MMP-2, are important modulators in the inflammatory reactions and healing process in IBD, such as interleukin (IL)-1, an important proinflammatory cytokine, fibroblast growth factor (FGF) receptor-1 ectodomain, which modulates the angiogenic activity of FGF, inactivated monocyte chemoattractant protein (MCP)-3, all affecting the inflammatory response.[36,37] 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.[18,20,22] 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.[19,22,32] Immunohistochemically, however, either no,[16] diffuse (present study) or diverse positive cells[22] were seen. One of the main reasons of this inconsistency is probably the sequestration of MMP-2 to the matrix components, which prohibits an exact localization.[38] Similar to the study by von Lampe *et al.*[19], 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 remodelling 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 tumour 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.[10] 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.[10,39] Thus, the production of MMP-2 seems to be delicately regulated during inflammatory and healing processes in IBD.

In general, the constitutive expression of MMPs is rather low, with the exception of the neutrophil MMPs MMP-8 and MMP-9.[4,5] It is believed that MMP-9 is stored in the secondary and tertiary granules of neutrophils for rapid release into inflammatory sites.[5] We found the amount of MMP-9 protein to be increased in relation to inflammation in full thickness IBD mucosal 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* hybridisation for mRNA.[15,18,22] 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.[32] 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 difference from the inflamed tissue.[18] 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,[18] as exemplified by increased myeloperoxidase levels.[40] In general, the studies demonstrate that MMP-9 participates actively in the inflammatory process of IBD, especially in the acute phase. Our BIA analysis also revealed an enhanced MMP-9 activity in association with severity of inflammation of the tissue in CD as well as, and most prominently, in UC. Apparently, this enhanced MMP-9 activity in severely inflamed IBD tissue is not counteracted by the simultaneously twofold increased level of TIMP-1 (unpublished observation). 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.[3,5,8,41] The ECM fragments produced by the activation of MMP-9 are also known to be chemotactic for inflammatory cells.[7] In a previous study, we also showed that TNF-α is an important regulator of the transcription of MMP-9 mRNA in blood leukocytes in patients with CD.[42] Furthermore, Sanceau *et al.*[43] reported that TNF-α can activate the MMP-9 gene transcription through regulation of the nuclear factor (NF)-κB activation. Recently, Kirkegaard *et al.*[22] reported that MMP-9 contributes to fistula formation in CD through degradation of the ECM, whereas, it has been reported that MMP-9 has a function in tissue repair by facilitating re-epithelialisation and degradation of denatured collagen.[30] Thus, similar to MMP-2, MMP-9 also seems to contribute to the different phases of the intestinal inflammatory process, from damage to repair, which is not specific for IBD and has also been observed in other inflammatory processes and even in malignant diseases.[3-8] 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 CD 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.

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