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

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Chapter 5

Increased Mucosal Matrix Metalloproteinase-1, -2, -3 and -9 Activity in Patients with Inflammatory Bowel Disease and the Relation with Crohn's Disease Phenotype

Short title: MMP activity in IBD

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Abstract

Background/aims. Matrix metalloproteinases are associated with matrix turnover in both physiological and pathological conditions. We postulate an association between aberrant matrix metalloproteinases proteolytic activity and the intestinal tissue destruction, seen in patients with Crohn's disease and/or ulcerative colitis.

Methods. Surgically resected inflamed and non-inflamed ileum and colon with/without extensive fibrosis from 122 Crohn's disease, 20 ulcerative colitis and 62 control patients were homogenized. Protein levels of matrix metalloproteinases and tissue inhibitor of metalloproteinases were measured by enzyme-linked immunosorbent assays (ELISA), while matrix metalloproteinases and myeloperoxidase activity were measured by specific activity assays.

Results. Expression of total levels of matrix metalloproteinases-1, -2, -3 and -9 relative to tissue inhibitor of metalloproteinases-1 and -2 was increased in inflamed inflammatory bowel disease compared to non-inflamed inflammatory bowel disease and control intestinal mucosa. Also, net matrix metalloproteinases-1, -2, -3 and -9 activity in inflamed inflammatory bowel disease was increased, with similar expression profiles in Crohn's disease and ulcerative colitis. Within inflamed inflammatory bowel disease, a close correlation of matrix metalloproteinases with myeloperoxidase was observed. The expression of matrix metalloproteinases and tissue inhibitor of metalloproteinases was similar in inflamed Crohn's disease tissue with or without extensive fibrosis and not related to fistulizing disease.

Conclusions. We have shown increased net matrix metalloproteinases activity in intestinal inflammatory bowel disease tissue, likely to contribute to the tissue damage and remodelling seen in inflammatory bowel disease.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the major forms of chronic idiopathic inflammatory bowel disease (IBD). CD is characterized by periodic, segmental and often transmural infiltration of potentially the whole gastrointestinal tract, especially the ileocaecal area, by a variety of immune cells like T and B lymphocytes, histiocytes (developing into granulomas/giant cells) and granulocytes.[1] The inflammatory infiltrate and resident cells secrete pro-inflammatory chemokines and cytokines, proteinases, reactive oxygen radicals, etc., which cause extensive morphological damage manifested by ulcera, abcesses, fissures and fistulae. In a subset of patients the sustained intestinal inflammation activates (myo)fibroblasts and smooth muscle cells to deposit massive amounts of collagen III and V, resulting in fibrotic strictures, halting food passage and necessitating surgical removal of the affected bowel.[2,3] Idiopathic UC affects the superficial mucosal layers of the colon, often starting from the rectum and extending proximally over the years.[4] Although stenotic development is not as common as with CD, in UC the inflammatory infiltrate may also result in extensive mucosal damage, with surgical removal of part or the whole colon as the final clinical outcome.

The matrix metalloproteinases (MMP) are a family of calcium and zinc containing neutral endoproteinases implicated in matrix tissue turnover during normal growth, development and reproduction but are also involved in several pathological conditions, i.e. cancer metastasis, rheumatoid arthritis, atherosclerosis, psoriasis, etc.[5] They have been shown to degrade a considerable number of important structural matrix molecules (Table 1), and an altered production of MMPs might contribute to the tissue morphological changes seen in IBD patients. Actually, reports have demonstrated an increased level of several MMPs in inflamed intestine of IBD patients, which was accompanied by an insufficient upregulation of the endogenous MMP inhibitors, i.e., tissue inhibitor of metalloproteinases (TIMP).[6-14] These studies do not report, however, about the actual activity of these MMPs in the IBD tissues. Also, in several models of IBD, e.g., DSS- and TNBS-induced colitis in rats and mice, immune infiltration was clearly associated with an upregulation of MMPs and the mucosal damage could be reversed by application

of specific MMP inhibitors.[15-17] However, in fibrotic and stenotic areas, the increased synthesis of collagens by mesenchymal cells may actually lead to a thickening of the bowel wall probably because of dysregulated MMP matrix degradative capacity. For example, in fistulae with chronic inflammation and fibrosis only a moderate upregulation of MMP-3 and -9 versus TIMP was observed opposite to the massive upregulation of these MMP members in areas with acute inflammation without fibrosis.[18] Given the potential relevance in IBD pathogenesis, their interactions and substrate diversity (Table 1), we not only measured MMP-1, -2, -3, -9 and TIMP-1, -2 protein levels by ELISA but also their activity by specific immunocapture bioactivity assays. We show an upregulation of these MMPs relative to TIMP, associated with higher (patho)physiological MMP activity in inflamed IBD intestinal mucosa compared with non-inflamed tissue from both IBD and control patients. In CD patients, MMP levels were independent of the macroscopical/histological co-presence of fibrosis and were not related to the incidence of fistulae during follow-up.

Table 1. Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) analyzed in this study, with their characteristics.[5,38]

MMP/TIMP	Synonym(s)	Major Substrate(s)	Molecular weight(s) (kDa[a])
MMP-1	Interstitial collagenase Collagenase-1	Fibrillar collagens, pro-MMP-2 and -9	52 and 41
MMP-2	Gelatinase A	Collagens, gelatins, elastins, laminin	72 and 66
MMP-3	Stromelysin-1	Extracellular glycoproteins, non-fibrillar collagens, pro-MMPs	57, 45 and 28
MMP-9	Gelatinase B	Collagens, gelatins, elastins, laminin	92 and 85
TIMP-1	Fibroblast collagenase inhibitor Collagenase inhibitor	(pro-) MMP-9	28
TIMP-2	CSC-21K	(pro-) MMP-2	21

[a]kDa = kilo Dalton

Materials and methods

Tissue samples

Over the period 1983–2002 macroscopically inflamed/affected as well as non-inflamed/unaffected intestinal mucosa was prospectively collected immediately after surgical resection from 122 (49 male (M)/73 female (F)) CD and 20 (6 M/14 F) UC patients at the departments of Surgery, Pathology and Gastroenterology-Hepatology of our hospital and stored at –70 ^{0}C (Table 2). The IBD patients underwent operation because of stricturing processes, fistulae and/or luminal disease activity refractory to medical therapy (aminosalicylates, corticosteroids, azathioprine, methotrexate and/or anti-TNF-α antibody infliximab). Severe fibrosis in inflamed CD mucosa, often manifested by a thickened wall and narrowed lumen, was documented in the histopathology reports from the Pathology department. Incidence of peri-anal, entero–entero, entero–viscero and/or enterocutaneous fistulae in CD patients in their clinical history and during follow-up (evaluation period median 24.7, range 3.3–58.5 years) was recorded in patient files from the Gastroenterology department. Also, from 62 (26 M/36 F) colorectal carcinoma or adenoma patients, macroscopically normal control mucosa at least 10 cm away from the surgically resected neoplasia was collected. Reflecting the early onset, CD and UC compared to control patients were younger at time of surgery (Table 2). Intestinal mucosa was homogenized in 1 ml 0.1 M Tris–HCl, 0.1% Tween 80, pH 7.5 per 60 mg tissue using a Potter device (B Braun, Germany) as described previously.[19] Protein concentration was determined by the method of Lowry *et al*.[20] and myeloperoxidase (MPO) activity was measured as described elsewhere, based on the conversion of the ortho-dianisidine dihydrochloride MPO substrate in the presence of hydrogen peroxide.[21]

Determination of MMP and TIMP by ELISA and BIA

Levels of MMP-2 and -9 protein were measured in appropriately diluted homogenates by our in-house ELISAs, as described previously.[8] TIMP-1 and -2 proteins were measured by commercially available ELISAs from R&D systems, according to the manufacturer's instructions.[22] MMP-1, -2, -3, and -9 activities were determined by highly specific immunosorbent activity assays (BIAs) from

Table 2. Patient and tissue specifications

	CD	UC	IBD	Controls	*P*-value[a]
# Patients (♂, ♀)	122 (49, 73)	20 (6, 14)	142 (55, 87)	62 (26, 36)	0.67
# Inflamed tissue (ileum, colon)	197 (109, 73)[b]	36 colon	233 (109,109)[b]	0	0.014
# Non-inflamed tissue (ileum, colon)	145 (88, 48)[b]	15 (4, 11)	160 (92, 59)[b]	72 (24, 48)	<0.001
# Averaged inflamed tissue[d]	155	24	179	0	NA
Medication (mild, strong, unknown)[c]	51, 90, 14	NA	51, 90, 38	NA	NA
# Averaged non-inflamed tissue[d]	125	15	140	62	NA
Medication (mild, strong, unknown)[c]	47, 66, 12	NA	47, 66, 27	NA	NA
Median age at surgery (range)	36.6 (11.6–78.7)	32.4 (19.2–64.7)	36.1 (11.6–78.7)	52.9 (19.0–85.0)	<0.001

NA = not applicable.

a *P*-value IBD vs. controls, note the relative abundance of ileum relative to colon tissue and young age at surgery in IBD.

b In addition, 15 inflamed and 9 non-inflamed CD tissues with unspecified intestinal origin were collected.

c Mild treatment: patients received no medication or were treated with mesalazine; Strong treatment: corticosteroids, azathioprine, infliximab, etc.

d Protein measurements on multiple tissue resection specimens with similar inflammation status and collected at the same surgery were averaged.

Amersham Biosciences, essentially as described elsewhere.[8,23,24] In brief, sample MMPs were captured by immobilized MMP mono-specific antibodies in microtitre plates. After incubation in buffer with (for total MMP activity) or without (for endogenously active MMP) latent MMP activating *p*-aminophenyl mercuric acetate (APMA), pro-urokinase modified to contain a MMP recognition cleavage site was added. The MMP-activated pro-urokinase subsequently converted peptide substrate S-2444 and absorption was measured at 405 nm. Results are expressed as arbitrary units/mg protein.

Statistical analysis

Kolmogorov–Smirnov analysis of data sets revealed statistically significant deviation from normal distribution, thus unpaired and paired differences were assessed by non-parametric Mann–Whitney *U*-tests and Wilcoxon signed-ranks tests, respectively. Correlation between two variables was performed by Spearman ranks correlation test or Pearson *Chi*-square test. Differences and correlations were deemed statistically significant when two-tailed *P*-value ≤0.05. All tests were performed using SPSS statistical software, version 11.0.

Results

A consistent upregulation of MMP protein, along with MPO activity, was observed in inflamed IBD intestinal mucosa (Fig. 1). Total levels of MMP-1 activity (BIA with APMA) and MMP-2 and -9 protein were upregulated 2.5-, 1.8- and 6.8-fold compared to controls, respectively, while the overexpression of total MMP-3 activity was even more impressive (18.6 versus 0.0 U/mg). The increase in total MMP protein/activity was only partially compensated by TIMPs: TIMP-1 was upregulated 1.7-fold, while TIMP-2 levels were similar between IBD and control tissue. When compared to (paired) non-inflamed IBD tissue, MMPs and TIMP-1 were 1.8–7.8 versus 1.9-fold upregulated, respectively, while non-inflamed IBD compared to control mucosa expressed higher levels of MMP-3 and -9 (2.4 versus 0.0 U/mg and 9.7 versus 5.5 ng/mg) along with a small increase in MPO activity. The magnitude of the overexpression of MMP relative to TIMP in inflamed IBD is shown in Table 3. The increase in absolute *and* relative MMP and TIMP levels was paralleled by an increase in *net* (BIA without APMA) MMP activity in inflamed IBD versus control tissue (Fig 2: MMP-1, -2, -3 and -9: 5.6 versus 2.7, 1.2 versus 0, 5.7 versus 0, 181 versus 65 U/mg, respectively) and in net MMP-3 activity compared to non-inflamed IBD tissue. In non-inflamed IBD versus control tissue, the relative expression of MMP-3 and MMP-9 to TIMP was increased as well (Table 3), but this was not sufficient to significantly increase their net MMP activity (Fig. 2). Expression of MMP, TIMP and MPO appeared very similar in CD compared to UC with corresponding inflammation status; only in inflamed CD tissue total MMP-1

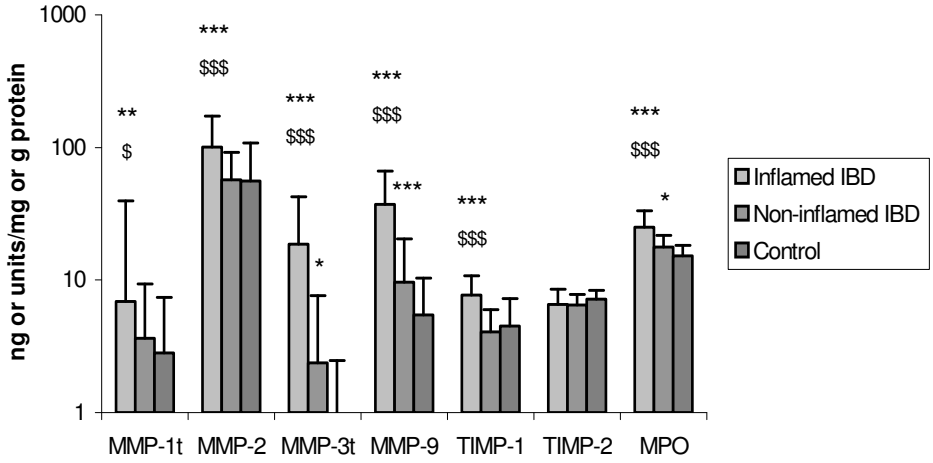

Figure 1. Total MMP, TIMP and MPO protein/activity expression (median + 75th percentile) by inflamed and non-inflamed IBD vs. normal control intestinal mucosa. *n* = 28, 25, 16 (MMP-1t); 70, 50, 22 (MMP-3t); 178–179, 140, 62 (MPO, MMP-2, -9 and TIMP-1, -2) for inflamed IBD, non-inflamed IBD and controls, respectively. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. control; $^{\$\$\$}P < 0.001$, $^{\$\$}P < 0.01$, $^{\$}P < 0.05$ vs. paired non-inflamed IBD (*n* = 25, 47 and 127–128 for MMP-1, -3t and MMP-2, -9, TIMP-1, -2, MPO, respectively). MMP-1 and -3 activities (U/mg protein) were measured by BIA (+APMA) and MMP-2, -9 and TIMP-1,-2 (ng/mg) by ELISA, respectively. Note the logarithmic scale on the *y*-axis.

activity was found to be lower [respectively, 5.9 (0.0–120.7) versus 23.0 (0.0–187.4) U/mg, $P < 0.05$] whereas TIMP-2 was found to be higher [respectively, 6.7 (2.1–16.2) versus 5.5 (3.2–10.20) ng/mg, $P < 0.01$]. In inflamed CD, most of these protein markers were slightly downregulated when patients were treated with strong medication compared with mild medication prior to surgery, while these findings were essentially reproduced in non-inflamed CD (data not shown). The distribution of ileal and colonic tissues is different between the IBD and control groups and therefore the altered expression of MMPs in IBD might thus be attributed to a higher proportion of ileum tissue in this group (Table 2). However, when the above analyses were repeated stratified according to tissue origin, i.e., ileum and colon, similar results were obtained (data not shown).

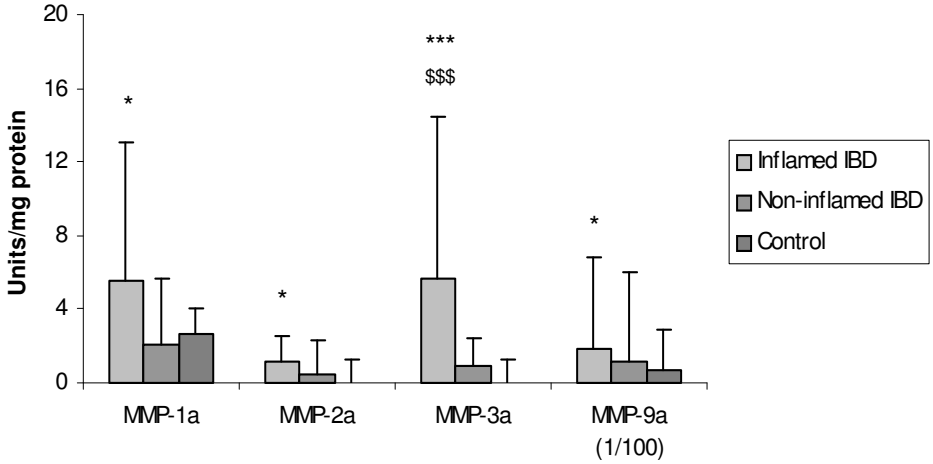

Figure 2. Net MMP activity (median + 75th percentile) in inflamed and non-inflamed IBD vs. macroscopically normal control intestinal mucosa. n = 28, 25, 16 (MMP-1a); 70, 52, 22 (MMP-2, -3, -9a) for inflamed IBD, non-inflamed IBD and controls, respectively. $^{***}P < 0.001$, $^{*}P < 0.05$ vs. controls; $^{\$\$\$}P < 0.001$ vs. paired non-inflamed IBD (n = 25 for MMP-1 and n = 49 for MMP-2, -3 and -9a). MMP activities (U/mg protein) were measured by BIA with omission of APMA. For graphical purposes, actual MMP-9 values are 100-fold larger than depicted.

Within the inflamed IBD tissues, expression of MMPs and TIMPs was often correlated to MPO activity. Notably, the net MMP-3 activity was strongly correlated with MPO, as were the total MMP-3/TIMP ratios ($0.41 \leq \rho \leq 0.47$, $P < 0.001$). Also, net MMP-1 activity was associated with MPO ($\rho = 0.38$, $P < 0.05$), but the levels of total MMP-1/TIMP were not. Remarkably, net gelatinase activities were not correlated with MPO, while the MMP-9, but not the MMP-2, and the MMP-9/TIMP ratios were ($0.42 \leq \rho \leq 0.47$, $P < 0.001$). Finally, no difference in expression of any of the MMP, TIMP or MPO parameters was observed in inflamed CD tissue in relation to the co-presence of fibrosis and none of these markers were related to the incidence of fistulae during follow-up (Fig. 3a and b).

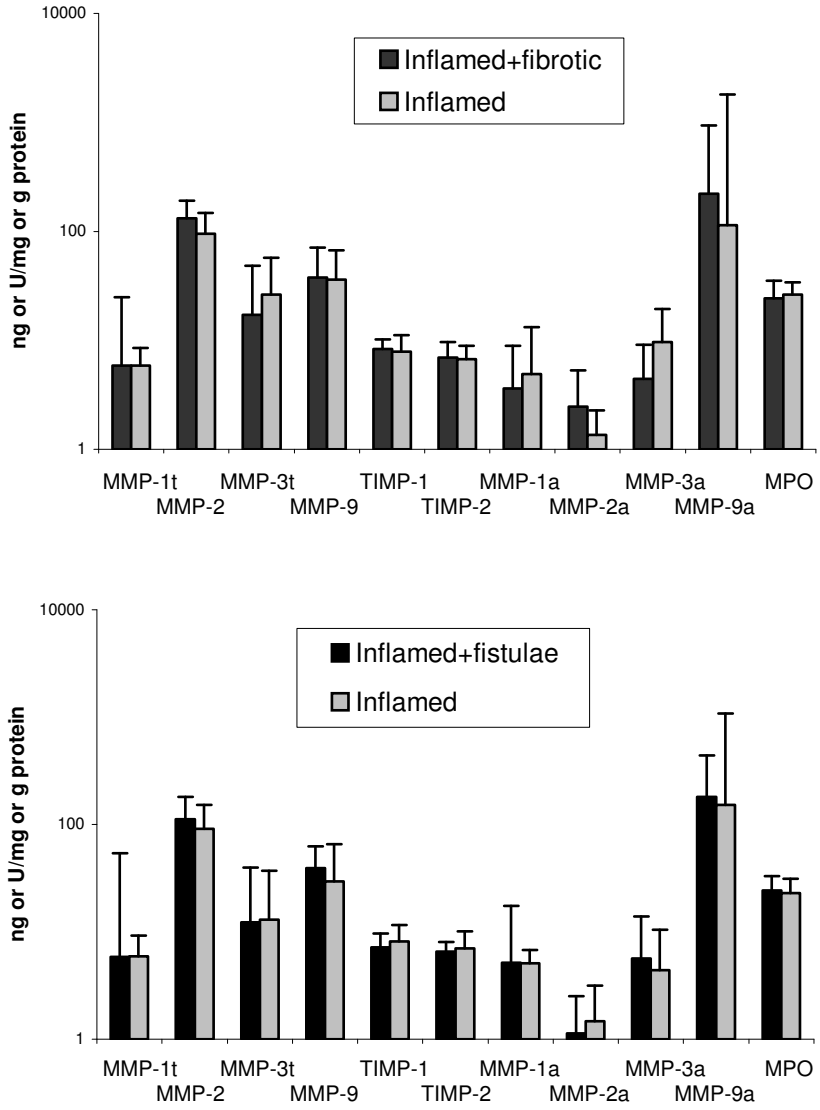

Figure 3. MMP, TIMP and MPO protein/activity expression (median + 75th percentile) by inflamed CD intestinal mucosa with or without extensive fibrosis (a) and with or without fistulizing disease during follow-up (b) (unpaired data). *n* = 7–10 (MMP-1t, MMP-1a); 16–31 (MMP-2a, MMP-3t, MMP-3a, MMP-9a); 40–103 (MPO, MMP-2, -9 and TIMP-1, -2). Note the logarithmic scale on the *y*-axis.

Table 3. Median (range) MMP over TIMP ratios in inflamed and non-inflamed IBD vs. control intestinal mucosa.

Ratio	Inflamed IBD	Non-inflamed IBD	Controls
MMP-1/TIMP-1	1.1 (0.0–14.3)	0.7 (0.0–23.4)	0.6 (0.0–21.0)
MMP-1/TIMP-2	1.5 (0.0–38.2)**	0.7 (0.0–35.9)	0.4 (0.0–10.7)
MMP-2/TIMP-1	12.0 (0.8–87.8)	12.2 (2.3–1787.6)	11.8 (2.0–85.5)
MMP-2/TIMP-2	14.4 (0.8–90.7)***,†	8.1 (1.1–117.0)	8.1 (2.1–88.9)
MMP-3/TIMP-1	2.3 (0.0–22.2)***,†	0.5 (0.0–6.8)*	0.0 (0.0–0.9)
MMP-3/TIMP-2	2.8 (0.0–66.3)***,†	0.3 (0.0–23.5)*	0.0 (0.0–0.6)
MMP-9/TIMP-1	4.3 (0.2–33.0)***,†	2.5 (0.1–259.0)***	1.3 (0.1–7.5)
MMP-9/TIMP-2	5.1 (0.2–67.2)***,†	1.5 (0.1–100.9)***	0.9 (0.1–5.1)

n = 28, 25, 16 (MMP-1); 70, 50, 22 (MMP-3) and 179, 139-140, 62 (MMP-2, -9), respectively. MMP-1 and -3 were measured in BIA with APMA and ratios to TIMP are in U/ng, MMP-2 and -9 were measured in ELISA and ratios have no unit.* $P < 0.05$ vs. controls.** $P < 0.01$ vs. controls.*** $P < 0.001$ vs. controls.† $P < 0.001$ vs. paired non-inflamed IBD (*n* = 25 (MMP-1), 47 (MMP-3), 127-128 (MMP-2, -9)).

Discussion

We have shown an upregulation of MMP-1, -2, -3 and -9 protein relative to TIMP-1, -2 in inflamed CD and UC versus non-inflamed IBD and normal intestinal mucosa, while non-inflamed IBD already contained more MMP-3 and -9 relative to TIMP compared to control tissue. More importantly, however, also the net activity of MMP-1, -2, -3 and -9 was increased in inflamed IBD and within this group, MMP-1 and -3 activities were correlated with MPO level. There are several mechanisms by which the inflammatory infiltrate might induce net MMP activity. The cytokines secreted by lymphocytes, monocytes and neutrophils, i.e., TNF-α, IL-1β, IFN-γ, etc., have been shown to induce synthesis of MMPs relative to TIMP not only in the inflammatory cells themselves, but also in the resident cells.[25-27] The induced MMPs might be activated by the MPO generated chlorinated oxidants,[28] while these radicals were also shown to degrade TIMP.[29] In addition, MMP-14, the natural activator of MMP-2, seems to be upregulated in IBD,[14] leading to increased

active MMP-2 and the latter can proteolytically activate MMP-9.[30] Also, the increased MPO activity likely reflects an inflammatory infiltrate at least partly consisting of neutrophils, and neutrophil-derived elastase was shown to degrade TIMP and activate MMP.[31] As mentioned before, MMP-1, -2, -3 and -9 collectively are capable of cleaving various structural matrix molecules. Also, they have been shown to cleave several non-structural proteins as well, for instance, MMP-9 potentiates pro-inflammatory interleukin-8 by removing the first six aminoterminal aminoacids[32] and processes IL-1β into an active form,[33] while MMP-2 and MMP-9 both degrade substance P, an important neurokine.[34,35] Furthermore, MMP-1 degrades IGFBP thus releasing IGF mitogenic activity,[36] while MMP-3 inactivates α(2)-antiplasmin, thus favouring local plasmin mediated proteolysis.[37] In combination, the increased levels of these MMPs probably mediate not only the structural damage to the tissue but may also propagate the excessive immune response seen in intestinal tissue from IBD patients. Importantly, the medication our patients received, whether mesalazine, corticosteroids, azathioprine, etc., could not prevent the increase in MMP over TIMP. This is in support of our hypothesis of MMP-mediated tissue damage necessitating surgery. However, some of the high MMP protein/activity levels we observed in inflamed IBD tissue may also counteract tissue damage. For instance, MMP-2 was demonstrated to protect against colitis probably by increasing intestinal barrier function.[38] In inflamed CD tissue with versus without extensive fibrosis, MMP and TIMP protein/activity levels were found to be similar, corroborating the results obtained by Warnaar *et al*.[39] These authors also demonstrated increased MMP expression in the healthy proximal margin of resected ileal CD tissue by immunohistochemistry and on the mRNA level, suggesting an active role for MMPs in fibrosis, perhaps in facilitating (myo)fibroblast migration.[40] This hypothesis is confirmed by the inhibition of bleomycin-induced pulmonary fibrosis in mice and of TNBS-induced colonic strictures in rats by the MMP inhibitors batimastat® and phenantroline, respectively.[16,41] Alternatively, the MMPs might intentionally counteract the process of fibrosis but may simply not be sufficiently upregulated to degrade the excessive collagen deposition.[18,42,43] In our study, the MMP levels in resected inflamed (and non-inflamed, not shown) CD tissue were not related to the incidence of fistulae

during follow-up. In a previous report, Kirkegaard *et* al.[18] demonstrated increased MMP levels within fistulae of CD patients. These observations indicate that the inflammatory process is accompanied by increased MMP proteolytic activity but that is not indicative as such for the fistulizing phenotype of CD. In that respect we recently also noticed that infliximab treatment of *ex vivo* explants of inflamed intestinal tissue from IBD patients decreased MMP-1, -3 and -9 protein and activity relative to the TIMPs, but not that of MMP-2, which in patients with CD was also reflected in the serum during treatment follow-up.[44,45] In summary, our study extends previous reports by showing an association between upregulated levels of MMP-1, -2,-3, -9 and morphological damage, i.e., ulcers and also stenotic strictures in intestinal tissue from IBD patients, not only at the MMP protein but particularly at the activity level. Future studies should focus on proof of *in vivo* activity, for instance by detection of proteolytic fragments likely to originate from cleavage by MMP, which could be followed by trials involving administration of specific MMP (-1, -3, -9) inhibitors to IBD patients suffering from relapse.

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