Original Article

Differential Expression and Processing of Matrix Metalloproteinase 19 Marks Progression of Gastrointestinal Diseases

(matrix metalloproteinase 19 / inflammatory bowel disease / macrophages / colon cancer / endothelium / lymphatic vessels)

M. ČERVINKOVÁ[1], P. HORÁK[2], I. KANCHEV[1], R. MATĚJ[3], J. FANTA[2], R. SEQUENS[4], P. KAŠPÁREK[1,5], L. SARNOVÁ[1], J. TUREČKOVÁ[1], R. SEDLÁČEK[1]

[1]Laboratory of Transgenic Models of Diseases, Institute of Molecular Genetics of the ASCR, v. v. i., Prague, Czech Republic
[2]Department of Surgery, First Faculty of Medicine, Charles University in Prague and Na Bulovce Hospital, Prague, Czech Republic
[3]Department of Pathology and Molecular Medicine, Thomayer Hospital, Prague, Czech Republic
[4]Gastroenterology Surgical Centre, Hospital of Merciful Sisters of St. Borromeo, Prague, Czech Republic
[5]Department of Genetics and Microbiology, Faculty of Sciences, Charles University in Prague, Czech Republic

Abstract. Matrix metalloproteinases (MMPs), responsible for extracellular matrix remodelling and processing of numerous soluble and cell-surface proteins, appear to play important roles in pathogenesis of gastrointestinal diseases. MMPs influence migration of inflammatory cells, mucosal destruction, matrix deposition and degradation. In this study, we analysed the expression of MMP-19 in the main forms of gastrointestinal diseases including inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease, and colorectal carcinoma. We identified prominent MMP-19 expression in unaffected areas of intestinal epithelia and macrophages but not in other cells or tissues. Abundant expression of MMP-19 was also found in the endothelium of blood and lymphatic vessels of inflamed intestinal tissue. High MMP-19 immunoreactivity was also associated with macrophages in inflamed areas and myenteric plexuses. In comparison to the intestinal epithelium, all these cell types and compartments appeared to express MMP-19 irrespective of the disease pathogenesis and progression. Intestinal epithelia exhibited striking differential immunoreactivity for MMP-19. While immunoreactivity of monoclonal antibody recognizing the propeptide domain declined in virtually all IBD and colorectal carcinoma samples, other polyclonal antibodies against the hinge region and propetide domain did not show such an obvious decrease. Additional Western blotting analysis revealed that the antibodies against MMP-19 recognize differently processed forms of this MMP. The disappearance of immunoreactivity of the monoclonal anti-propeptide domain antibody does not mean down-regulation of MMP-19, but processing of the immature form. As this processing likely leads to the activation of this MMP, the differential staining pattern may be an important sign of disease progression.

Received November 26, 2013. Accepted January 10, 2014.

Financial support was given to RS by GACR (P302/11/2048 and P303/10/2044), the project BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University (CZ.1.05/1.1.00/02.0109; European Regional Development Fund), GAUK (project 64212) and OPVK (CZ.1.07/2.3.00/20.0102). The work was institutionally supported by RVO: 68378050.

Corresponding author: Radislav Sedláček, Laboratory of Transgenic Models of Diseases, Institute of Molecular Genetics of the ASCR, v. v. i., Vídeňská 1083, 142 20 Prague 4, Czech Republic. Phone: (+420) 24106 3137; Fax: (+420) 22431 0955; e-mail: radislav.sedlacek@img.cas.cz.

Abbreviations: CD – cluster of differentiation, CRC – colorectal cancer, DAB – diaminobenzidine, ECM – extracellular matrix, EGFP – enhanced green fluorescent protein, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, HUVEC – human umbilical vein endothelial cells, IBD – inflammatory bowel disease, IGF – insulin-like growth factor, IGFBP – insulin-like growth factor-binding protein, MMP – matrix metalloproteinase, PFA – paraformaldehyde, qRT-PCR – quantitative reverse transcriptase polymerase chain reaction, RT – room temperature.

Introduction

Inflammatory bowel diseases (IBDs) and colon cancer, which are thought to be associated with western life

style and whose aetiopathogeny still remains unclear, have shown continuously increasing incidence over the last decade (Ponder and Long, 2013). Deregulation of matrix metalloproteinases (MMPs) that are associated with these diseases is thought to be co-responsible for the development of intestinal inflammation and cancer as administration of broad-spectrum metalloproteinase inhibitors reduces inflammation and disease severity in mouse models of colon injury (Naito et al, 2004; Wang and Wang, 2008). Under normal conditions, the majority of MMPs are present at low levels and are responsible for normal tissue homeostasis since their well-controlled activity is important for physiologic processes including morphogenetic events during development such as blood vessel growth and mammary gland development (Vu and Werb, 2000; Page-McCaw et al., 2007) or physiologic tissue maintenance and repair such as wound healing and repair, reproductive processes, and innate immune reactions (Curry and Osteen, 2003; Parks et al., 2004; Cox et al., 2008; Dean et al., 2008; Khokha et al., 2013).

The human *MMP19* gene is located on chromosome 12 mapping to the 14q region (Pendás et al., 1997). Whereas most other MMPs are encoded by 10 exons, human and murine MMP-19 are encoded by nine exons. The human proenzyme consists of 508 amino acids and has a molecular weight of 57.4 kDa. MMP-19 is, like most other members of the MMP family, secreted as a zymogene and displays the typical structural features of MMPs. The proteinase consists of a signal peptide, a propeptide domain, a catalytical domain, a hinge region, and a haemopexin-like domain. MMP-19 contains two putative N-glycosylation sites (Mueller et al., 2000). Despite the typical domain structure, MMP-19 displays a striking difference in the auto-inhibitory part of the sequence within the propeptide domain. The consensus sequence PRCGLED is believed to enable the autocatalytic activation of MMP-19 (Stracke et al., 2000b).

In contrast, altered expression and dysregulation of MMP activities are associated with the development of chronic inflammatory diseases as well as cancerogenesis (Meijer et al., 2007; Roy et al., 2009). In IBD, MMPs appear to be involved in all stages of the disease development including tissue remodelling found during mucosa destruction, which leads to inflammation, ulceration, and fistula formation in Crohn's disease as well as to influx of inflammatory cells into the inflamed area (Pender and MacDonald, 2004; Naito and Yoshikawa, 2005; Ravi et al., 2007). While some MMPs, such as MMP-1, -3, -7, -10, -12, -19, -26, and -28, are expressed in the intestine under non-inflammatory conditions, a number of publications report on up-regulation and changed expression of MMPs expressed by immune cells, intestinal epithelium, and fibroblasts in inflammatory bowel disease (Bister et al., 2004; Medina and Radomski, 2006; Monteleone et al., 2006; Pedersen et al., 2009) and colorectal cancer (Roy et al., 2009; Hadler-Olsen et al., 2013). Generally, MMPs were studied as potential prognostic biomarkers or targets for therapeutic agents; nevertheless, their expression and impact on gastrointestinal diseases is not completely understood.

In this study, we focused on the comparison of expression patterns of MMP-19 in the main diseases of human gastrointestinal pathologies as understanding of MMP-19 expression and function is still incomplete. MMP-19 shows a definite expression pattern in several cell types that are substantially involved in IBD and colorectal cancer. It exhibits strong and inducible expression in macrophages (Sedlacek et al., 1998; Mauch et al., 2002) and capillary endothelial cells in acute inflammation area in rheumatoid arthritis (Kolb et al., 1997, 1999). MMP-19 also cleaves components of ECM, especially basement membrane, including laminin 5 γ2 chain, nidogen-1, tenascin C, collagen IV, and aggrecan (Stracke et al., 2000a,b; Titz et al., 2004; Sadowski et al., 2005) as well as some secreted proteins. Processing of plasminogen by MMP-19 mediates the anti-angiogenic effect in endothelial cells (EC) via generation of angiostatin-like fragments, and the MMP-19-dependent cleavage of IGFBP-3 appears to regulate IGF-1-mediated proliferation, migration, and adhesion of keratinocytes (Sadowski et al., 2003; Brauer et al., 2011). The epithelial and epidermal role of MMP-19 could be inferred from down-regulation of MMP-19 in tumours and anti-tumorigenic effect (Djonov et al., 2001; Jost et al., 2006; Chan et al., 2011).

The biological role of MMP-19 is also being deciphered from studies on MMP-19-deficient mice, which exhibit reduced development kinetics of thymocytes and low contact hypersensitivity response (Beck et al., 2008). MMP-19 activity also influences the fibrotic process in the lung and liver as the knockout mice show a hepatoprotective effect through diminished replacement of the physiological extracellular matrix with fibrotic and enhanced lung fibrosis following bleomycin treatment (Jirouskova et al., 2012; Yu et al., 2012).

In this study, we compared the expression of MMP-19 in ulcerative colitis and Crohn's disease with that of colorectal cancer using immunohistochemistry. The study revealed that MMP-19 is generally expressed in macrophages, myenteric plexus (Cajal cells), and endothelial lining of blood vessels and weakly in lymphatic vessels. An interesting differential expression pattern was revealed in intestinal epithelia as monoclonal anti-propeptide antibody as well as polyclonal anti-hinge and anti-propeptide antibodies exhibit differential staining: while the immunoreactivity of monoclonal antibody against the propeptide domain decreases strikingly or is virtually absent, the staining by the polyclonal ones remains largely unchanged in both IBD and colorectal cancer. Additional analysis using cell culture experiments showed that MMP-19 undergoes differential processing that is likely responsible for this differential pattern. Thus, the decline of immunoreactivity against the propeptide domain, i.e. unprocessed form of this MMP, likely marks the development of intestinal diseases, having thus broader meaning for understanding the in-

volvement of MMPs in gastrointestinal and other diseases.

Material and Methods

Clinical material

The study was approved by the ethics committee before its commencement and informed consents were signed by patients before the surgical procedure provided. The IBD cohort contained nine patients afflicted by ulcerative colitis, eight by fibrostenosing type of Crohn's disease and five by fistulous (penetrating) type of Crohn's disease. Six of these patients suffered from diverticulitis/diverticulosis in steady state of the disease; two of them exhibited intestinal ischaemia, which reasoned for surgical intervention. Five patients with histological evidence of colorectal cancer were included in the colorectal cancer (CRC) group. All were diagnosed with colorectal adenocarcinoma, no metastases, grade II-III (Table 1). In the control group of tissues, samples were taken from a healthy area, i.e. non-malignant and non-inflammatory tissues, of small intestine and colon. All samples were larger resections; no biopsies were included as they do not comprise the whole colonic wall.

Immunohistochemistry

Formalin-fixed and paraffin-embedded samples were cut into 2 µm sections and stained with haematoxylin and eosin (HE) for initial morphology evaluation. Immunohistochemistry was done with serial sections acquired from every single sample. The primary antibodies against the following proteins were used: MMP-19 (raised against residues surrounding glycine 44; NB100-91875, Novus Biologicals, Littleton, CO), MMP-19 (against the hinge region; ab38966, Abcam, Cambridge, UK), MMP-19 (against propeptide, CK8/4, own production) (Mauch et al., 2002), CD31 (Dako, Glostrup, Denmark), CD68 (Zytomed, Berlin, Germany), and podoplanin (Dako). Details are given in Table 2.

Table 1. Patients

Group	Age (years)	Gender (male/female)
Control	47-69	4/4
IBD	21-77	13/9
CRC	36-74	4/1

After rehydration all slides were retrieved in a pressure cooker for 15 min at pH 9 (for CD68, MMP-19 CK8/4, MMP-19 NB100-91875 and MMP-19 ab38966) and pH 6 (CD31 and podoplanin). The endogenous peroxidase activity was quenched in 3% (v/v) methanol solution of hydrogen peroxide for 20 min at room temperature (RT). Unspecific binding was blocked by using 2% (w/v) BSA solution for 20 min at RT. All primary antibodies were applied for 1 h at RT.

Detection of the primary immune complexes was done by incubation with secondary EnVision™ system (Dako) employing anti-mouse or rabbit ready-to-use peroxidase-conjugated polymers, which were incubated on the sections for 40 min at RT. The reaction was developed via DAB+ kit (Dako) prepared according to the manufacturer's instructions and incubated for 10 min at RT. The development was stopped by flushing the slides with distilled water, followed by counterstaining in Harris' haematoxyllin. All slides were mounted in Aquatex™ (Merck-Millipore, Darmstadt, Germany). Negative controls were performed by omitting the primary antibody. Analysis was performed in a Leica DM3000 microscope system (Leica Microsystems, Leitz, Germany) equipped with a Leica DFC450 colour camera (Leica Microsystems). All representative images were acquired in the same system and post-processed in Adobe Photoshop (Adobe Systems Inc, San Jose, CA).

Normalized semi-quantitative score of the samples was done according to the following formula:

$$\frac{N1x0 + N2x1 + N3x2 + N4x3}{N1 + N2 + N3 + N4} /100 =$$

= normalized score (scoring value 0–3)

where: N1 is the number of cells/structures exhibiting no immune reactivity/negative (scored as 0), N2 is the number of cell/structures exhibiting low intensity of immune reactivity (scored as 1); N3 is the number of cells/structures exhibiting moderate intensity of immune reactivity (scored as 2), and N4 is the number of cells/structures having high intensity of the immune reactivity (scored as 3). In the respective study, the intensity/number of the gut-lining epithelium, macrophages and plexuses was scored by the number of cells; hence the lymphatic vessels and the peripheral vasculature was scored as the number of positive vessels. We have chosen the following criteria for positive vessels: at least

Table 2. Antibodies for immunohistochemistry

Target	Dilution	Clone	Manufacturer	Catalogue #
CD31	1 : 100	JC70A	Dako, Glostrup, Denmark	M0823
CD68	1 : 500	514H12	Zytomed, Berlin, Germany	603-1332
MMP-19	1 : 100	polyclonal	Novus Biologicals, Littleton, CO	NB100-91875
MMP-19	1 : 500	polyclonal	Abcam, Cambridge, UK	ab38966
MMP-19	1 : 200	CK8/4	own production	-
podoplanin	1 : 200	D2-40	Dako, Glostrup, Denmark	M3619

50 % of the vessels trans, longitudinal or transverse section should exhibit immune reactivity.

Expression analysis

Total RNA was isolated from the dissected tissue or cell cultures using TriReagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. RNA concentration was determined using a Nanodrop ND-1000 instrument (Thermo Scientific, Wilmington, DE). Unique primers were designed for ~100 bp segments of target gene transcripts using Primer-BLAST online software: MMP-19 forward GCCAAAGCTCGTACTGTTCC, reverse CCCTCAGTCCAGAACTCGTC; GAPDH forward GTGAAGGTCGGAGTCAACG, reverse TGAGGTCAATGAAGGGGTC; PPIA forward GCCGAGGAAAACCGTGTACT, reverse CTTTGGGACCTTGTCTGCAA. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was carried out as described (Chalupský et al., 2013), directly from isolated RNA using Kapa SYBR Fast One-Step qRT-PCR Kit (Kapa Biosystems, Boston, MA) in LightCycler 480 (Roche, Mannheim, Germany). The relative mRNA levels were calculated by comparative Ct method (Buryova et al., 2013) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or peptidyl-prolyl *cis-trans* isomerase (PPIA) as the control.

Cell culture, transfection, and immunoblotting analysis

Immortalized human epithelial colorectal adenocarcinoma cell line Caco-2, human epithelial colorectal cancer cell line HCT116, and human umbilical vein endothelial cell (HUVEC) line were purchased from American Type Culture Collection (ATCC, Manassas, VA). Caco-2 and HCT116 cells were both grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich), HUVEC were cultivated in F-12K medium (Gibco Life Technologies, Carlsbad, CA). All media were supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin/streptomycin (both PAA Laboratories, Colbe, Germany), and F-12K medium was in addition supplemented with endothelial cell growth supplement (ECGS; Sigma-Aldrich). The cells were kept under standard cell culture conditions (37 °C, 5% CO_2) and used at passage numbers not higher than five. Transient transfections of HCT116 cells were carried out in serum-free media using X-tremeGENE HP (Roche) according to the manufacturer's instructions.

Protein precipitates, obtained in parallel to RNA isolation (see above), were dissolved in 1% SDS and protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL). Proteins were separated in 10% SDS gels (25 μg per lane) and transferred to a nitrocellulose membrane (Whatman, Maidstone, UK). Immunoblots were probed with antibodies against MMP-19 (as above; see Immunohistochemistry paragraph) at the following concentrations: 1 : 500 (NB100-91875), 1 : 3000 (CK8/4), and 1 : 5000 (ab38966). Secondary antibodies, goat anti-mouse (1 : 10,000; Pierce) and goat anti-rabbit (1 : 10,000; Jackson ImmunoResearch, Newmarket, UK), were peroxidase-conjugated. Signals were detected using ECL plus Western Blotting Detection System (Cell Signaling Technology, Boston, MA) and recorded with a luminescent image analyser (LAS-3000, Fujifilm Life Science, Düsseldorf, Germany).

Results

Expression pattern of MMP-19 in small intestinal and colon wall

To describe the expression pattern of MMP-19 in diseased small intestine and colon, samples from IBD and colon cancer patients (for details see Table 1) were compared to control samples histologically assigned as healthy or with signs of mild inflammatory reaction from patients with diverticulitis/diverticulosis or patients with ischemic ileus. These samples were obtained from the small intestine (N = 9) or from colon (N = 10). Samples of diseased tissue either with chronic or acute IBD were dissected from the small intestine, which exhibited Crohn's disease in all cases (N = 19). Colon samples contained ones with ulcerative colitis (N = 11) and Crohn's disease (N = 3), colorectal cancer samples from the areas neighbouring tumours (N = 6) or containing tumour tissue itself (N = 3). Immunostaining for MMP-19 with monoclonal antibody CK8/4 recognizing an epitope in the propeptide domain (Mauch et al., 2002) revealed the expression pattern restricted to several anatomical structures and cell types. One of the most prominent staining patterns was identified in the blood and lymphatic vessels (Fig. 1 A,E,B,F). Blood vessels in the mucosa and submucosa as well as the vessels in the muscle layer and subserosal exhibited strong MMP-19 immunoreactivity in endothelial cells (Fig. 1 A,E). Differences in intensity among the arteries and capillaries were not obvious. This staining was prominent in all IBD and CRC samples as well as in control samples that were used for comparison to IBD and CRC. Lymphatic vessels were stained in all samples but showed rather weaker immunoreactivity than the endothelial lining in the blood vessels (Fig. 1 A,B,E,F).

Other cells showing prominent immunoreactivity to MMP-19 were macrophages, which were reported to express this MMP previously (Mauch et al., 2002). They appeared to express MMP-19 in all samples, although IBD samples exhibiting inflammatory reaction showed increased numbers of positive macrophages (Fig. 1 C,G), especially macrophages in IBD as well as granuloma (not shown). Although fibroblasts in samples from non-diseased areas were not stained by any of the used antibodies against MMP-19, samples from CRC exhibited abundant positive intestinal fibroblasts (Fig. 2).

Myenteric plexuses also exhibited abundant immunoreactivity to MMP-19 (Fig. 1 D,H). Primarily higher intensity was observed in the Cajal cell compartment,

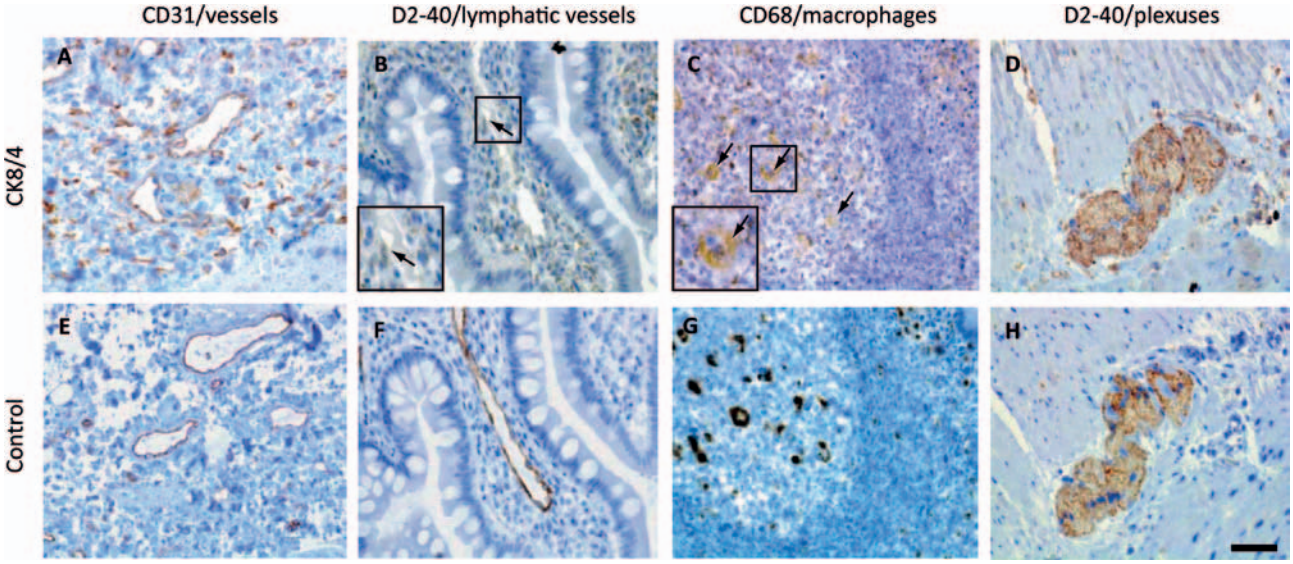

Fig. 1. The expression pattern of MMP-19 in intestinal and colon walls
All antibodies used revealed the identical staining pattern; images shown in the top panel were obtained using the monoclonal antibody CK8/4. The staining shows moderate to strong levels in peripheral vasculature endothelium (**A**), slight expression in lymphatic vessels, primarily in the endothelium (**B**, arrow and insert), macrophages (**C**, arrows and insert) endothelium and myenteric plexuses (**D**). To identify the structures stained for MMP-19, the following antibodies were used as controls: CD31 for peripheral blood vasculature endothelium (**E**), podoplanin for lymphatic vessels (**F**), CD68 for macrophages (**G**), and myenteric plexuses with Cajal cells were identified using haematoxylin and eosin staining (**H**). Original magnification 40×. Bar represents 50 μm.

and somewhat weaker staining was detected in some neurocytes. The last compartment exhibiting intensive anti-MMP-19 staining was intestinal epithelium (Fig. 2), which showed differential staining intensities among the sections exhibiting IBD and CRC and sections from unaffected areas. Quantitative data on immunolabelled structures are summarized in Table 3.

Expression of MMP-19 in gut epithelium

Staining with monoclonal antibody CK8/4 revealed moderate to strong signal intensity in small intestinal and colon epithelium in samples from non-inflamed epithelium (Fig. 2 A). Enterocytes in crypts and villous epithelium generally did not show differences in the staining although the staining appeared to be predominantly localized to the basal and apical sites of epithelial cells. In contrast to these samples, epithelia in IBD and colorectal carcinoma samples showed dramatic reduction of staining (CRC) or its absence (IBD) (Fig. 2).

As the monoclonal antibody CK8/4 specifically recognizes a linear epitope at the propeptide domain (Mauch et al., 2002), which is typically cleaved off during the process of activation or any processing, i.e. the antibody primarily recognizes an unprocessed form of this MMP, we used another two antibodies to find out whether MMP-19 is down-regulated or whether the signal disappears due to the processing. Both of these polyclonal antibodies recognized the recombinant MMP-19 in a similar way as CK8/4, although the band pattern was not identical (Fig. 3). Histochemistry staining employing these polyclonal antibodies against the hinge region (ab38966) and propeptide (NB100-91875) exhibited a similar staining pattern as that of CK8/4, i.e. they recognized all cell types and structures as CK8/4 antibody; however, their immunoreactivity did not disappear in IBD samples, pointing to possible processing of MMP-19 (Fig. 2).

To perform analysis of MMP-19 expression in IBD and colorectal cancer samples at the transcriptional level, mRNA was isolated and qRT PCR was performed. For the purpose of analysis, healthy and afflicted tissue intestinal samples were classified and divided into the following groups:

i) non-inflammatory Crohn's disease (CD-NI)
ii) inflammatory Crohn's disease (CD-I)
iii) ulcerative colitis (UC) (Fig. 4 A)

or

i) non-inflammatory Crohn's disease (CD-NI)
ii) inflammatory samples with preserved mucosa, Crohn's disease (non-lesion; CD-INL)
iii) inflammatory parts where mucosa was lost, Crohn's disease (lesion; CD-IL).

As the comparison of individual classified groups showed significant differences (Fig. 4), the differential staining of epithelia likely indicates rather the processing of MMP-19 than a change in expression levels, which might mean an important point in the progression of intestinal diseases.

In vitro validation of MMP-19 forms by antibodies

In order to confirm the finding that the differential staining pattern of anti-MMP-19 antibodies in intestinal

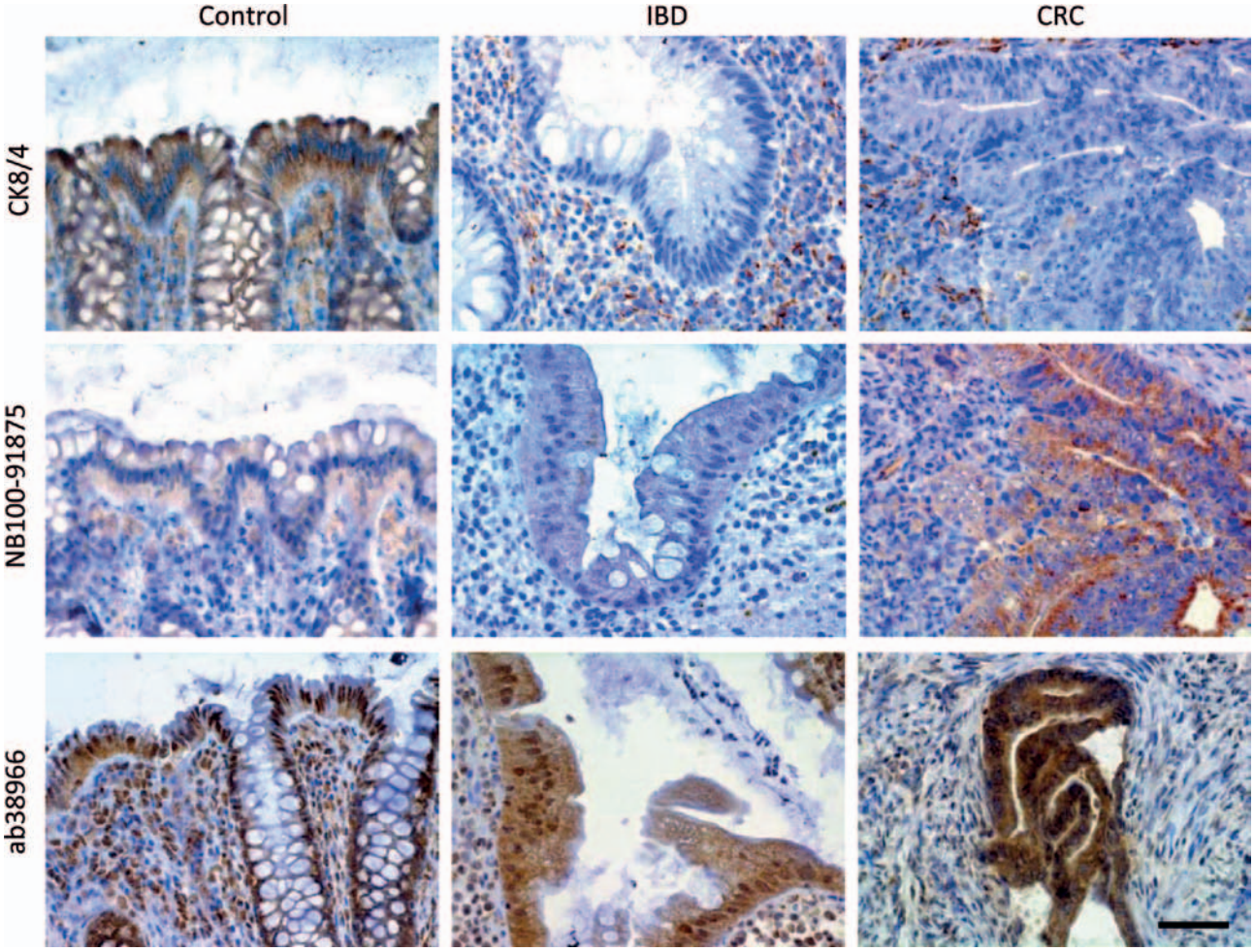

Fig. 2. Differential staining pattern of MMP-19 in the colon epithelia
Staining patterns in control, IBD, and CRC samples were acquired by three different antibodies against MMP-19: polyclonal antibodies (ab38966 and partially NB100-91875) showed an increased epithelial IHC signal in both IBD and CRC compared to control samples. The most positive staining was seen in CRC. A similar pattern was observed in sections stained with the polyclonal antibodies against the MMP-19 hinge region (ab38966); increased epithelial IHC intensity can be seen in IBD and the highest in CRC samples compared to the control ones. The monoclonal antibody (CK8/4) gives strong signal in the control samples and decreased or absent signal in IBD and is completely absent in CRC samples. Control, healthy tissue.
Original magnification 40×. Bar represents 50 μm.

Table 3. Immunoreactivity of samples and different interstitial structures/cell types. Numbers are given as stained and scored samples/available samples (immune reactivity). Immune reactivity was scored 0–3, where: 0 – no staining, 1 – low intensity of staining, 2 – moderate intensity of staining, 3 – high intensity of staining (see also Material and Methods section).

Group	Lymphatic vessels	Vessels	Macrophages	Plexuses
Control	9/9 (0-2)	9/9 (0-2)	9/9 (0-3)	9/9 (0-3)
IBD	26/26 (0-2)	26/26 (0-2)	26/26 (0-3)	24*/26 (0-3)
CRC	4/4 (0-2)	4/4 (0-2)	4/4 (0-3)	0/4*

*Plexuses either swollen or not present in the evaluated sections

epithelium is based on recognition of different MMP-19 forms, the antibodies used were tested *in vitro* for their ability to recognize distinct MMP-19 fragments by immunoblotting. First, antibodies were tested for their ability to recognize the recombinant N-terminal fragment of MMP-19 (proΔ260–508MMP-19) comprising the propeptide and the catalytic domain (Fig. 3). According to expectation, the anti-hinge polyclonal antibodies ab38966 did not recognize this MMP-19 form. The recognition pattern was also analysed in cell lysates prepared from the endothelial HUVEC cell line and epithelial HCT116 cells, which ectopically expressed N-ter-

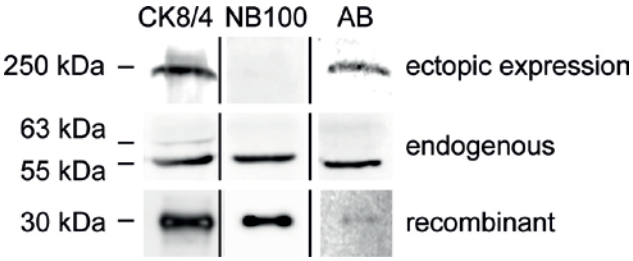

Fig. 3. In vitro recognition of different MMP-19 forms
HTC116 cells ectopically expressing EGFP-tagged MMP-19 (ectopic expression) and HUVEC cells (endogenous) were lysed and together with the recombinant N-terminal fragment of MMP-19 (proΔ260–508MMP-19; recombinant) separated in SDS-10% PAGE gel, transferred onto nitrocellulose membrane and visualized using CK8/4, NB100-91875 (NB100) and ab38966 (AB) antibodies.

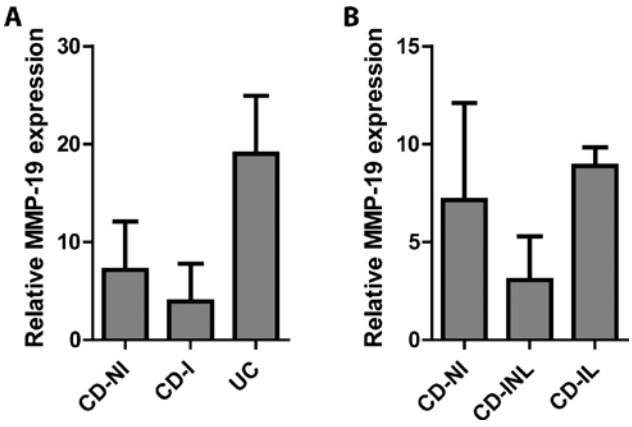

Fig. 4. Relative expression of MMP-19 in healthy and afflicted intestinal wall
Tissue samples from either non-inflammatory (CD-NI), inflammatory (CD-I) intestinal samples of patients with Crohn's disease and ulcerative colitis (UC; **A**) or non-inflammatory parts (CD-NI), inflammatory samples with preserved mucosa (non-lesion; CD-INL) and inflammatory parts where mucosa was lost (lesion; CD-IL) of Crohn's disease-afflicted small intestine (**B**) were processed and relative expression levels of MMP-19 were determined by qRT-PCR. MMP-19 expression was normalized to GAPDH.

minally tagged MMP-19-EGFP (Fig. 3). Monoclonal CK8/4 antibody recognized bands corresponding to the ectopically expressed EGFP-tagged full-length MMP-19 (250 kDa) and recombinant proΔ260–508MMP-19 (30 kDa; Fig. 3). The EGFP-tagged full-length MMP-19 in supernatants migrated in SDS-PAGE at 250 kDa, likely due to the BSA content. Similarly to the polyclonal NB100-91875 and ab38966 antibodies, CK8/4 recognized a 55 kDa-band corresponding to the fragment of endogenous MMP-19. In addition, CK8/4 also detected a larger fragment (~63 kDa), possibly corresponding to the unprocessed, full-length and possibly glycosylated form (Fig. 3). Polyclonal anti-hinge ab38966 antibodies recognized EGFP-tagged full-length MMP-19 as well as recombinant proΔ260–508MMP-19, although they detected only the 55 kDa but not the 63 kDa form of endogenous MMP-19 in the lysate from HUVECs. Finally, polyclonal NB100-91875 antibodies recognized both 55 and 30 kDa forms of MMP-19 but could not detect the full-length MMP-19 (Fig. 3).

Discussion

MMPs are involved in virtually all pathophysiologic remodelling processes. Alteration in their expression and activity participates in the development and progression of gastrointestinal diseases such as IBD and colorectal cancer. In this study, we intended to obtain a complete image of the expression pattern of MMP-19 in the small intestine and colon and find out whether the MMP-19 staining pattern in the epithelia is changing among the stages found in healthy, IBD, and cancer tissues. The reason for this study was the incompletely characterized expression and role of MMP-19 in the major forms of gastrointestinal pathologies. Previously published data using uncharacterized antibodies showed that MMP-19 might be expressed in epithelial cells of healthy intestinal tissue and up-regulated in the inflamed regions in fibroblasts, macrophages, shedding epithelium and in areas of epithelial proliferation, and that it is down-regulated in the epithelia of colon cancer (Bister et al., 2004). Our findings are partially in concordance with the previously reported data.

However, in addition, we identified MMP-19 expression in the endothelial lining of blood and lymphatic vessels and cells of myenteric plexuses, especially in Cajal cells. MMP-19 in blood and lymphatic vessels was detected generally in all samples irrespective of the disease state. The expression in endothelium of blood vessels is in agreement with our previously published data showing expression of MMP-19 in endothelial cells (Kolb et al., 1997, 1999), especially in endothelial cell of capillaries and small vessels of inflamed regions but not in larger arteries and veins. The expression of MMP-19 in the endothelial lining of lymphatic vessels was observed for the first time. Although the function of this MMP in lymphatic vessels is unknown, it could have a similar function as proposed for MMP-19 in the blood vessels, i.e. this proteinase with the ability to cleave components of the basement membrane (Stracke et al., 2000b; Titz et al., 2004; Sadowski et al., 2005) could be involved in the growth of vessels.

The expression of MMP-19 in myenteric plexuses, especially in interstitial cells of Cajal cells (ICC), was observed for the first time. The role of ICC remains unclear (Rumessen et al., 2011), especially in inflammatory bowel disease and colorectal cancer, although ICC are likely to be involved in motoric activity of the gastrointestinal tract (Sanders et al., 2010). The role of MMP-19, as well as of any other MMP, in myenteric plexuses might be seen in its proteolytic activity as it could be involved in damage to enteric nerves and neuromuscular junctions (Wang et al., 2007) or in remodelling of the myenteric plexus extracellular matrix.

The most intriguing phenomenon found in this study was the differential staining patterns of antibodies recognizing distinct epitopes of MMP-19. This differential staining might represent a hallmark of the disease progression due to the changes in MMP-19 processing and likely also its activity. We observed a disappearance or decrease of anti-MMP-19 staining using the monoclonal anti-propeptide-specific antibody in the tissues from IBD and colorectal cancer patients. The decrease of MMP-19 staining or expression in colorectal cancer was also reported previously (Bister et al., 2004).

Moreover, other reports also showed a decline or down-regulation of MMP-19 in neoplastic dedifferentiation in breast cancer and in the development of nasopharyngeal carcinoma (Chan et al., 2011; Djonov et al., 2001). Other reports have documented up-regulation of MMP-19 in the cancer development. Thus, MMP-19 is highly expressed in proliferating astrocytoma and glioma cells, and it also hallmarks progression of cutaneous melanoma, promoting invasion of tumour cells (Lettau et al., 2010; Muller et al., 2010). Thus, the scenario of regulation and expression of an MMP might be dependent on the cell type as well as on the type of the disease, e.g. cancer versus inflammation. The expression of MMP-19 at the RNA level did not significantly alter in IBD samples as evidenced by qRT PCR analysis. This is consistent with our ChIP-seq data generated from colonic cancer samples, which showed no significant differences between different patient groups (data not shown). Therefore, to further analyse the expression pattern and processing of MMP-19, we employed two additional antibodies recognizing the propeptide domain and the hinge region of MMP-19, respectively.

The immunoblotting analysis showed clear differences in the recognition of MMP-19 forms and fragments. In general, it appears that the monoclonal antibody CK8/4 recognizing the propeptide domain (Mauch et al., 2002) cannot detect MMP-19 once the propeptide domain is cleaved off, while the anti-hinge antibodies also recognize the N-terminally processed forms of MMP-19. The second type of polyclonal antibodies, although also specific for the majority of MMP-19 recombinant protein forms, show rather an intermittent staining pattern, which might be due to broader or distinct epitopes recognized in MMP-19. As the monoclonal antibody CK8/4 did not stain the processed form of MMP-19 in samples of gastrointestinal diseases, whereas the polyclonal antibodies, recognizing rather the processed forms of MMP-19, still stained the majority of these samples, we concluded that MMP-19 was not primarily down-regulated. The removal of the propeptide domain from MMP zymogens is a typical step in their activation (Van Wart and Birkedal-Hansen, 1990) This processing likely leads to MMP-19 activation, which seems to mark progression of the gastrointestinal diseases studied.

The disappearance of the staining of monoclonal anti-propeptide MMP-19 antibody in combination with the staining of other antibodies might thus be important in recognizing the disease progression; this change would mean that MMP-19 is processed and/or activated with or without involvement of additional activating protease. One of the unique features of MMP-19 is the altered latency motif, which normally ensures inactive state, but MMP-19 autoactivates rapidly (Sedlacek et al., 1998; Stracke et al., 2000b). It is also possible that other MMPs and proteases might be involved in the processing and activation of MMP-19 as the whole family of MMPs acts, together with other proteases, in the proteolytic activating cascade (Nagase et al., 2006).

Although the role and all substrates of MMP-19 in the intestine have not yet been elucidated in the gut homeostasis and development of colorectal diseases, this study shows that MMP-19 is likely to be involved in the progression of colorectal diseases and that the differential staining pattern mirroring MMP-19 processing may mark progression of colorectal as well as other diseases.

Acknowledgement

We thank Attila Juhász and Markéta Pícková for their outstanding technical assistance.

References

Beck, I. M., Ruckert, R., Brandt, K., Mueller, M. S., Sadowski, T., Brauer, R., Schirmacher, P., Mentlein, R., Sedlacek, R. (2008) MMP19 is essential for T cell development and T cell-mediated cutaneous immune responses. *PLoS One* **3**, e2343.

Bister, V. O., Salmela, M. T., Karjalainen-Lindsberg, M. L., Uria, J., Lohi, J., Puolakkainen, P., Lopez-Otin, C., Saarialho-Kere, U. (2004) Differential expression of three matrix metalloproteinases, MMP-19, MMP-26, and MMP-28, in normal and inflamed intestine and colon cancer. *Dig. Dis. Sci.* **49,** 653-661.

Brauer, R., Beck, I. M., Roderfeld, M., Roeb, E., Sedlacek, R. (2011) Matrix metalloproteinase-19 inhibits growth of endothelial cells by generating angiostatin-like fragments from plasminogen. *BMC Biochem.* **12,** 38.

Buryova, H., Chalupsky, K., Zbodakova, O., Kanchev, I., Jirouskova, M., Gregor, M., Sedlacek, R. (2013) Liver protective effect of ursodeoxycholic acid includes regulation of ADAM17 activity. *BMC Gastroenterol.* **13,** 155.

Chalupský K, Kanchev I, Žbodáková O, Buryová H, Jiroušková M, Kořínek V, Gregor M, Sedláček R (2013) ADAM10/17-dependent release of soluble c-Met correlates with hepatocellular damage. *Folia Biol. (Praha)* **59,** 76-86.

Chan, K. C., Ko, J. M., Lung, H. L., Sedlacek, R., Zhang, Z. F., Luo, D. Z., Feng, Z. B., Chen, S., Chen, H., Chan, K. W., Tsao, S. W., Chua, D. T., Zabarovsky, E. R., Stanbridge, E. J., Lung, M. L. (2011) Catalytic activity of matrix metalloproteinase-19 is essential for tumor suppressor and anti-angiogenic activities in nasopharyngeal carcinoma. *Int. J. Cancer* **129,** 1826-1837.

Cox, J. H., Dean, R. A., Roberts, C. R., Overall, C. M. (2008) Matrix metalloproteinase processing of CXCL11/I-TAC results in loss of chemoattractant activity and altered glycosaminoglycan binding. *J. Biol. Chem.* **283,** 19389-19399.

Curry, T. E., Jr., Osteen, K. G. (2003) The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocr. Rev.* **24,** 428-465.

Dean, R. A., Cox, J. H., Bellac, C. L., Doucet, A., Starr, A. E., Overall, C. M. (2008) Macrophage-specific metalloelastase (MMP-12) truncates and inactivates ELR+ CXC chemokines and generates CCL2, -7, -8, and -13 antagonists: potential role of the macrophage in terminating polymorphonuclear leukocyte influx. *Blood* **112,** 3455-3464.

Djonov, V., Hogger, K., Sedlacek, R., Laissue, J., Draeger, A. (2001) MMP-19: cellular localization of a novel metalloproteinase within normal breast tissue and mammary gland tumours. *J. Pathol.* **195,** 147-155.

Hadler-Olsen, E., Winberg, J. O., Uhlin-Hansen, L. (2013) Matrix metalloproteinases in cancer: their value as diagnostic and prognostic markers and therapeutic targets. *Tumour Biol.* **34,** 2041-2051.

Jirouskova, M., Zbodakova, O., Gregor, M., Chalupsky, K., Sarnova, L., Hajduch, M., Ehrmann, J., Jirkovska, M., Sedlacek, R. (2012) Hepatoprotective effect of MMP-19 deficiency in a mouse model of chronic liver fibrosis. *PLoS One* **7**, e46271.

Jost, M., Folgueras, A. R., Frerart, F., Pendas, A. M., Blacher, S., Houard, X., Berndt, S., Munaut, C., Cataldo, D., Alvarez, J., Melen-Lamalle, L., Foidart, J. M., Lopez-Otin, C., Noel, A. (2006) Earlier onset of tumoral angiogenesis in matrix metalloproteinase-19-deficient mice. *Cancer Res.* **66,** 5234-5241.

Khokha, R., Murthy, A., Weiss, A. (2013) Metalloproteinases and their natural inhibitors in inflammation and immunity. *Nat. Rev. Immunol.* **13,** 649-65

Kolb, C., Mauch, S., Peter, H. H., Krawinkel, U., Sedlacek, R. (1997) The matrix metalloproteinase RASI-1 is expressed in synovial blood vessels of a rheumatoid arthritis patient. *Immunol. Lett.* **57,** 83-88.

Kolb, C., Mauch, S., Krawinkel, U., Sedlacek, R. (1999) Matrix metalloproteinase-19 in capillary endothelial cells: expression in acutely, but not in chronically, inflamed synovium. *Exp. Cell Res.* **250,** 122-130.

Lettau, I., Hattermann, K., Held-Feindt, J., Brauer, R., Sedlacek, R., Mentlein, R. (2010) Matrix metalloproteinase-19 is highly expressed in astroglial tumors and promotes invasion of glioma cells. *J. Neuropathol. Exp. Neurol.* **69,** 215-223.

Mauch, S., Kolb, C., Kolb, B., Sadowski, T., Sedlacek, R. (2002) Matrix metalloproteinase-19 is expressed in myeloid cells in an adhesion-dependent manner and associates with the cell surface. *J. Immunol.* **168,** 1244-1251.

Medina, C., Radomski, M. W. (2006) Role of matrix metalloproteinases in intestinal inflammation. *J. Pharmacol. Exp. Ther.* **318,** 933-938.

Meijer, M. J., Mieremet-Ooms, M. A., van der Zon, A. M., van Duijn, W., van Hogezand, R. A., Sier, C. F., Hommes, D. W., Lamers, C. B., Verspaget, H. W. (2007) Increased mucosal matrix metalloproteinase-1, -2, -3 and -9 activity in patients with inflammatory bowel disease and the relation with Crohn's disease phenotype. *Dig. Liver Dis.* **39,** 733-739.

Monteleone, G., Caruso, R., Fina, D., Peluso, I., Gioia, V., Stolfi, C., Fantini, M. C., Caprioli, F., Tersigni, R., Alessandroni, L., MacDonald, T. T., Pallone, F. (2006) Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. *Gut* **55,** 1774-1780.

Mueller, M. S., Harnasch, M., Kolb, C., Kusch, J., Sadowski, T., Sedlacek, R (2000) The murine ortholog of matrix metalloproteinase 19: its cloning, gene organization, and expression. *Gene* **256**, 101-111.

Muller, M., Beck, I. M., Gadesmann, J., Karschuk, N., Paschen, A., Proksch, E., Djonov, V., Reiss, K., Sedlacek, R. (2010) MMP19 is upregulated during melanoma progression and increases invasion of melanoma cells. *Mod. Pathol.* **23,** 511-521.

Nagase, H., Visse, R., Murphy, G. (2006) Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc. Res.* **69**, 562-573.

Naito, Y., Takagi, T., Kuroda, M., Katada, K., Ichikawa, H., Kokura, S., Yoshida, N., Okanoue, T., Yoshikawa, T. (2004) An orally active matrix metalloproteinase inhibitor, ONO-4817, reduces dextran sulfate sodium-induced colitis in mice. *Inflamm. Res.* **53,** 462-468.

Naito, Y., Yoshikawa, T. (2005) Role of matrix metalloproteinases in inflammatory bowel disease. *Mol. Aspects Med.* **26,** 379-390.

Page-McCaw, A., Ewald, A. J., Werb, Z. (2007) Matrix metalloproteinases and the regulation of tissue remodelling. *Nat. Rev. Mol. Cell Biol.* **8**, 221-233.

Parks, W. C., Wilson, C. L., Lopez-Boado, Y. S. (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat. Rev. Immunol.* **4,** 617-629.

Pedersen, G., Saermark, T., Kirkegaard, T., Brynskov, J. (2009) Spontaneous and cytokine induced expression and activity of matrix metalloproteinases in human colonic epithelium. *Clin. Exp. Immunol.* **155,** 257-265.

Pendás, A. M., Knäuper, V., Puente, X. S., Llano, E., Mattei, M. G., Apte, S., Murphy, G., Lopez-Otin, C. (1997) Identification and characterization of a novel human matrix metalloproteinase with unique structural characteristics, chromosomal location, and tissue distribution. *J. Biol. Chem.* **272**, 4281-4286.

Pender, S. L., MacDonald, T. T. (2004) Matrix metalloproteinases and the gut – new roles for old enzymes. *Curr. Opin. Pharmacol.* **4**, 546-450.

Ponder, A., Long, M. D. (2013) A clinical review of recent findings in the epidemiology of inflammatory bowel disease. *Clin. Epidemiol.* **5**, 237-247.

Ravi, A., Garg, P., Sitaraman, S. V. (2007) Matrix metalloproteinases in inflammatory bowel disease: boon or a bane? *Inflamm. Bowel Dis.* **13,** 97-107.

Roy, R., Yang, J., Moses, M. A. (2009) Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer. *J. Clin. Oncol.* **27,** 5287-5297.

Rumessen, J. J., Vanderwinden, J. M., Horn, T. (2011) Crohn's disease: ultrastructure of interstitial cells in colonic myenteric plexus. *Cell Tissue Res.* **344,** 471-479.

Sadowski, T., Dietrich, S., Koschinsky, F., Sedlacek, R. (2003) Matrix metalloproteinase 19 regulates insulin-like growth factor-mediated proliferation, migration, and adhesion in human keratinocytes through proteolysis of insulin-like growth factor binding protein-3. *Mol. Biol. Cell* **14,** 4569-4580.

Sadowski, T., Dietrich, S., Koschinsky, F., Ludwig, A., Proksch, E., Titz, B., Sedlacek, R. (2005) Matrix metalloproteinase 19 processes the laminin 5 γ2 chain and induces epithelial cell migration. *Cell. Mol. Life Sci.* **62,** 870-880.

Sanders, K. M., Hwang, S. J., Ward, S. M. (2010) Neuroeffector apparatus in gastrointestinal smooth muscle organs. *J. Physiol.* **588,** 4621-4639.

Sedlacek, R., Mauch, S., Kolb, B., Schatzlein, C., Eibel, H., Peter, H. H., Schmitt, J., Krawinkel, U. (1998) Matrix metalloproteinase MMP-19 (RASI-1) is expressed on the surface of activated peripheral blood mononuclear cells and is detected as an autoantigen in rheumatoid arthritis. *Immunobiology* **198,** 408-423.

Stracke, J. O., Fosang, A. J., Last, K., Mercuri, F. A., Pendas, A. M., Llano, E., Perris, R., Di Cesare, P. E., Murphy, G., Knauper, V. (2000a) Matrix metalloproteinases 19 and 20 cleave aggrecan and cartilage oligomeric matrix protein (COMP). *FEBS Lett.* **478,** 52-56.

Stracke, J. O., Hutton, M., Stewart, M., Pendas, A. M., Smith, B., Lopez-Otin, C., Murphy, G., Knauper, V. (2000b) Biochemical characterization of the catalytic domain of human matrix metalloproteinase 19. Evidence for a role as a potent basement membrane degrading enzyme. *J. Biol. Chem.* **275,** 14809-14816.

Titz, B., Dietrich, S., Sadowski, T., Beck, C., Petersen, A., Sedlacek, R. (2004) Activity of MMP-19 inhibits capillary-like formation due to processing of nidogen-1. *Cell. Mol. Life Sci.* **61,** 1826-1833.

Van Wart, H. E., Birkedal-Hansen, H. (1990) The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. *Proc. Natl. Acad. Sci. USA* **87,** 5578-5582.

Vu, T. H., Werb, Z. (2000) Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev.* **14,** 2123-2133.

Wang, X. Y., Zarate, N., Soderholm, J. D., Bourgeois, J. M., Liu, L. W., Huizinga, J. D. (2007) Ultrastructural injury to interstitial cells of Cajal and communication with mast cells in Crohn's disease. *Neurogastroenterol. Motil.* **19**, 349-364.

Wang, Y. D., Wang, W. (2008) Protective effect of ilomastat on trinitrobenzenesulfonic acid-induced ulcerative colitis in rats. *World J. Gastroenterol.* **14**, 5683-5688.

Yu, G., Kovkarova-Naumovski, E., Jara, P., Parwani, A., Kass, D., Ruiz, V., Lopez-Otin, C., Rosas, I. O., Gibson, K. F., Cabrera, S., Ramirez, R., Yousem, S. A., Richards, T. J., Chensny, L. J., Selman, M., Kaminski, N., Pardo, A. (2012) MMP19 is a key regulator of lung fibrosis in mice and humans. *Am. J. Respir. Crit. Med.* **186**, 752-762.