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

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Expression, Regulation and Clinical Relevance

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ISBN: 978-90-9024077-0

Printed by Gildeprint Drukkerijen B.V., Enschede, The Netherlands

The work described in this thesis was performed at the Department of Gastroenterology and Hepatology of the Leiden University Medical Center (Leiden, The Netherlands). The project was partly financed by a grant from the Netherlands Digestive Diseases Foundation (WS 98-17)

Printing of this thesis was financially supported by the Section Experimental Gastroenterology (SEG) of the Netherlands Society of Gastroenterology (NVGE).

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PROEFSCHRIFT

ter verkrijging van de graad van Doctor aan de Universiteit Leiden,

op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op donderdag 23 april 2009

klokke 16.15 uur

door

Martin Jan-Willem Meijer

geboren te Amsterdam

in 1974

Promotiecommissie:

Promoter:	Prof. dr. C.B.H.W. Lamers
Co-promoter:	Dr. ir. H.W. Verspaget
Referent:	Prof. dr. A.S. Peña, Vrije Universiteit Amsterdam
Overige leden:	Prof. dr. A. van der Laarse
	Prof. dr. P.H.A. Quax
	Prof. dr. D.W. Hommes
	Dr. R.A. van Hogezand

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Abbreviations

APMA	*p*-amino-phenyl-mercuric acetate
ARMS	Amplification refractory mutational system
BIA	Bio-immuno activity assay
CD	Crohn's disease
CI	Confidence interval
CRC	Colorectal carcinoma
DSS	Dextran sodium sulphate
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
HR	Hazard ratio
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
HRP	Horse radish peroxidase
kDa	Kilo Dalton
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
NS	Not significant
PMNL	Polymorphonuclear leukocyte
PWM	Pokeweed mitogen
RFLP	Restriction fragment length polymorphism
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumor necrosis factor
UC	Ulcerative colitis

Chapter 1

Introduction

Inflammatory Bowel Disease - a major health problem

Inflammatory bowel disease (IBD), i.e., Crohn's disease (CD) and ulcerative colitis (UC), are characterized by an idiopathic, chronic and recurrent inflammation of the gastrointestinal tract. In CD, inflammation is segmental and transmural, often localized in (but not confined to) the ileocaecal area, while UC is limited to the mucosal lining of the colon, often starting in the rectum and extending in proximal direction through the years. In both CD and UC, inflammation may result in severe tissue damage, i.e., discontinuation of the epithelial border, ulcera, fissures, loss of circular folding, cobblestone appearance, fibrosis, stenosis and in CD also the formation of entero-entero, entero-viscero or entero-cutaneous fistulae. Abdominal pain, increased defecation frequency, bloody diarrhea, nausea, significant body weight loss and anemia related fatigue all contribute to the general malaise IBD patients often experience. Disease course may be complicated by osteoporosis, arthritis, ankylosing spondylitis, iritis, uveitis, erythema nodosom and primary sclerosing cholangitis (UC). Patients are at increased risk for developing colorectal carcinoma[1,2] and recently, IBD was associated with a higher incidence of adverse pregnancy outcomes.[3] First line of treatment consists of 5-ASA containing mesalasine/sulphasalazine or corticosteroid budesonide/prednisolone tablets, enema or suppository. In corticosteroid refractory patients disease modifying drugs azathioprine, cyclosporin, methotrexate or mycophenolate mofetil are administered. The therapeutic arsenal for CD patients has been expanded recently with biological agents specifically targeting TNF-α in the immune cascade. Chimeric (75% human/25% murine) infliximab and humanised adalumimab anti TNF-α antibodies have proven to be effective in the clinical setting for steroid dependent/refractory CD patients.[4-6] Disadvantages include the large placebo effect requiring further optimalization, host antibody response to infliximab and high medical costs.[7,8] Biological agents against IL-12, adhesion molecules and IL-6 receptors are promising new candidates but are not expected to enter the market soon.[9,10] Despite advances in treatment protocols, the natural history of the disease appears unmodified.[11,12] Cumulative 10 year surgical resection probability rates vary from 25-60 % in CD and 25% in UC patients and these percentages have remained similar for 4 decades although surgical resection rates within the first year of

diagnosis might be decreasing.[13-16] The socioeconomic burden remains high: in the United States total costs of IBD related healthcare are approximately 1 billion US dollar per year.[17] When work-productivity losses due to chronic disability are taken into account, total costs of IBD in the USA were estimated $ 5 billion in 2000.[18] Therefore, much effort is spent in research aimed at identifying the epidemiology, etiology and pathogenic mechanisms underlying IBD.

Inflammatory Bowel Disease - epidemiology & etiology

Annual incidence rates of CD in white residents of Western countries vary between 4-9 per 100,000, whereas the incidence of UC appears somewhat higher (9-14 per 100,000), with concordant prevalence rates of 130-175 (CD) and 240-275 (UC) per 100,000 persons.[16,19-22] Studies from Canada and New Zealand, however, reported higher incidence figures for CD compared to UC (14.6 versus 14.3 and 16.5 versus 7.6 per 100,000, respectively).[23,24] In both CD and UC, distribution of age at onset of disease is skewed, with an incidence peak between 20-30 years. Incidence and prevalence rates have risen after the Second World War, reaching the current plateau in the 1970s, affecting more people in urban versus rural areas.[25,26] People in white collar occupations appear more at risk compared to other groups in the population.[27] A north-south gradient has been postulated,[28] but in industrializing countries, incidence and prevalence of IBD are also increasing,[29] as are disease rates among Asian immigrants in Western countries.[30] Although disease concordance rates in monozygotic twins are high (CD: 60, UC: 20 %), pointing to a genetic influence, these sub maximum figures also suggest the involvement of one or several environmental factor(s) in the etiology of IBD.[31] Smoking was established as such a factor, worsening the prognosis in CD[32] and instead protective in UC.[33] Other studies have suggested an association of CD and/or UC with infectious agents such as *Mycobacterium avium sp. Paratuberculosis*,[34] invasive *Escherichia coli* strains[35,36] and measles virus infection or vaccination.[37-39] In case of *Helicobacter pylori*, a protective effect was suggested.[40] Married couples are at greater risk of sequentially contracting the disease after cohabitation.[41] Persons born in the winter months were more at risk for IBD[42] and onset of symptoms was also especially observed during this period, thus arguing for the

rationale of an infectious agent involved in the etiology of IBD,[43] although this remains to be confirmed.[44] Other studies have focused on an association with dietary food intake, such as breastfeeding, fast food, coca cola beverages and chocolate consumption.[45-47] Use of oral contraceptives,[48,49] menstrual cycle[50] and psychological distress[51] have also been implicated, again with inconclusive or even contradictory results.[52] Probably, the rise in incidence of IBD after the Second World War in Western and industrializing countries might be attributed to the introduction of better sanitary conditions, thus improving survival of susceptible individuals and/or shifting the development of the immune system towards hypersensitivity.[53-55] It should be noted that incidence and prevalence of asthma and diabetes type I have also increased, pointing to a more general promoting effect of western lifestyle on auto-immune disease.[56]

Several studies indicate genetic susceptibility in the etiology of IBD as well. For instance, IBD concordance rates are higher in monozygotic versus dizygotic twins,[31] IBD affecting multiple family members is frequently seen,[57] and incidence rate of IBD appears to be associated with ethnicity and religion group, as demonstrated by the increased prevalence in white Caucasian subjects and Ashkenazi Jews compared to blacks, Asians and Hispanics.[58-60] Early genome-wide association studies have identified (potential) IBD loci on chromosomes 1, 3, 4, 5, 6, 7, 10, 12, 14, 16, 19, 22 and X.[61-66] Subsequent fine-mapping has revealed the involvement of the nucleotide-binding oligomerisation protein 2 (NOD2)/ caspase activation and recruitment domain 15 (CARD15) gene on chromosome 16q12 in CD susceptibility,[67,68] which was confirmed in other studies.[69,70] Interestingly, single nucleotide polymorphisms (SNPs) within this gene were also found to be associated with asthma,[71] Blau syndrome,[72] increased mortality following sepsis[73] and allogeneic stem cell transplantation.[74,75] This landmark success and the increasing availability of high density SNP arrays led to a surge in genome wide research and to the identification of other loci strongly implicated in the pathogenesis of CD and/or UC, including the gene encoding a subunit for the IL-23 receptor (IL-23R) on chromosome 1p31,[76,77] genes involved in autophagy/breakdown of intracellular pathogens (autophagy related 16-like 1 gene (ATG16L1) on 2q37 and immunity related p47 guanosine triphosphatase murine

orholog (IRGM) on 5q33,[78-82] a gene desert on 5p13 regulating expression of the prostaglandin receptor EP4 (PTGER4) (83), the nel-like 1 precursor (NELL1) gene on 11p15[84] and just recently 3p21.31, NKX2-3, CCNY (CD and UC) and PTPN2, HERC2 and STAT3 (UC).[85] Importantly, these studies were conducted in Caucasian populations and not replicated in Asian IBD patients, reinforcing the pivotal role of ethnicity in this matter.[86,87] Other genes potentially involved in the susceptibility and/or phenotype of CD and/or UC are derived from candidate gene approach and include those encoding interleukin-1 receptor antagonist (IL-1RA), IL-2, IL-4, IL-10, IL-11, tumor necrosis factor-alpha (TNF-α), nuclear factor kappa B (NFκB), Toll like receptors (TLR), discs large homolog 5 (DLG-5), mucins, organic cation transporter-1 and -2 (OCTN-1, -2), mannan binding lectin (MBL), multidrug resistance 1 protein (MDR) and pregnane X receptor (PXR).[88-102] Occasionally, conflicting data were generated, probably due to the relatively small sample size often used, especially in the early studies.[103-106] Also, because of the nature of this approach, significant results might indicate the association of an adjacent predisposing gene in linkage disequilibrium with the examined gene, complicating this matter. However, it appears that, in cooperation with selected environmental stimuli, different sets of predisposing genes might give rise to essentially the same clinical disease manifestation, collectively called IBD. This is corroborated by the large number of mouse models, where targeting (i.e., IL-10) or overexpressing (i.e., TNF-α) of different genes leads to similar IBD like disease.[107-112]

Inflammatory Bowel Disease - pathogenic mechanisms

A widely accepted hypothesis states that IBD is an exaggerated immune response towards commensal bacteria in genetically susceptible individuals.[113] The recent rise in incidence of both forms of IBD might be attributed to increased exposure to so-called psychrotrophic bacteria by introduction of refrigerated food (cold chain hypothesis).[114] The concept of commensal bacteria playing a pathogenic role is corroborated by the increased frequency of anti-*Escherichia coli* outer membrane porin C, anti-flagellin and autoreactive mycobacterial HSP65 antibodies in CD and/or UC.[115-117] Flagellin specific T cells were able to cause fulminant colitis in an adoptive mouse transfer model.[118] Moreover, murine models for IBD do not

develop enteritis given a pathogen-free environment, although this was just recently challenged by the development of chronic ileitis in germ/free SAMP1/YitFc mice, suggesting that bacteria exacerbate disease but are not required for induction.[119] In CD patients, expression of anti-bacterial α-defensins HD5 and HD6 by ileal Paneth cells is reduced, especially in NOD-2 mutation carriers.[120] The levels of mucus forming proteins mucin3, 4, 5B and of sealing tight junction proteins claudin 5 and -8 are downregulated, whereas expression of pore-forming claudin 2 and rate of epithelial cell apoptosis are increased, resulting in impaired mucosal barrier function.[121,122] After epithelial injury, in CD patients an abnormally low neutrophil accumulation was observed compared to healthy controls, suggesting an impaired innate immune response.[123] The impaired mucosal barrier and/or the impaired innate immunity might result in overexposure to commensal bacteria, initiating and/or propagating the uncontrolled adaptive immune response seen in IBD. Importantly, IBD T-lymphocytes and neutrophils demonstrate increased resistance to apoptosis, thus sustaining the immune response.[124-127] Also, T cell regulatory (Treg) function might be insufficient to dampen the inflammatory reaction.[128] Both diseases are characterized by upregulation of pro-inflammatory cytokines (i.e., IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-15, -16, -17, -32, TNF-α, IFN-γ), chemokines (MIP1α, MIP1β, MIP3α, MCP1, MCP2, RANTES), neuropeptide substance P, growth factors (bFGF, VEGF, KGF), eicosanoid PGE2, corresponding receptors (TNF-R2, neurokinin-1/substance P receptor) and endothelial/leucocyte adhesion molecules (ICAM-1, selectins, LFA-1, α4β7 integrin/MAdCAM) in the (inflamed) intestinal mucosa, not compensated for by anti-inflammatory cytokines, soluble receptors and/or receptor antagonists (i.e., IL-10, TGF-β1, sTNFR, sIL1-RII, sgp130, IL-1RA).[129-156] However, some important differences are observed in the cytokine profile between CD and UC (upregulation of IL-12, IL-23 versus IL-13, respectively), reflecting the Th1 versus Th2 nature of the corresponding disease.[157-160] The cytokine expression in IBD is different in chronic versus early lesions, thus complicating this issue.[161,162] The upregulation of cytokines, chemokines, neuropeptides, growth factors, receptors and adhesion molecules chronically activates resident mesenchymal, epithelial and immune cells and continuously attracts new leucocytes from the peripheral circulation. In the

battle against their unknown targets, these cells may damage the intestinal tissue in various ways. T cell activated neutrophils and macrophages release massive amounts of harmful reactive oxygen metabolites by NADPH-dependent oxidative burst, targeting membrane lipids, protein and DNA, thus disrupting cellular structure at the molecular level and promoting malignancy.[163-166] An imbalanced anti-oxidant response in IBD patients may exacerbate disease.[167-169] Cytotoxic CD8+ T cells release pore forming perforin, proteolytic granzymes and/or express Fas ligand, triggering apoptosis in epithelial cells and disrupting mucosal barrier function.[170-172] Cytotoxic perforin releasing CD4+ T cells were demonstrated in CD[173,174] and activated complement in conjunction with IgG1 auto antibodies against tropomyosin isoform 5 may target epithelial cells in UC.[175,176] Increased expression of tissue remodeling neutrophil elastase by neutrophils and of chymase and tryptase by mast cells is associated with IBD.[177-179] Synthetic elastase and tryptase inhibitors were found beneficial in experimental colitis.[180,181] Concurrent attenuated induction of serine anti-proteases might exacerbate disease.[182] All activated cells also release specific members of the tissue remodeling Matrix Metalloproteinases (MMP), which are described below.

Matrix metalloproteinases - classification

Based on the catalytic group at the active center, five classes of proteases are recognized, i.e., serine-, threonine-, cysteine-, aspartic- and metallo-proteases, divided into clans and families based on protein folding and sequence similarity, see also http://merops.sanger.ac.uk/.[183] Clan MA of the metalloproteases is divided into subclans MA (M) and MA(E). Proteases designated to subclan M all contain a conserved methionine residue to the carboxy side of the active center, thus forming a characteristic loop or “Met turn” in the protein secondary structure, providing the base of the active cleft and are therefore called metzincins.[184] The metzincins are currently categorized into 12 families, each split into a variable number of subfamilies. Subfamily M12B contains the ADAMs (A Disintegrin And Metalloproteinase) and ADAMTSs (A Disintegrin-like And Metalloproteinase with Thrombospondin type 1 motifS).[185,186] It includes TACE (TNF-α Converting Enzyme, ADAM-17), which is important in releasing membrane-bound TNF-α and

TNF-R from the cell membrane[187] and ADAMTS-4 and -5, which cleave aggrecan in cartilage and might contribute to the structural damage seen in human arthritis.[188-190] Subfamily M10A, also called the matrixins, contains the matrix metalloproteinases (MMP). The human genome currently comprises 23 different MMPs, according to substrate specificity and protein structure subdivided into the collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT) MMPs, and a rest group (Table 1, page 24-26).

Matrix metalloproteinases - structure

All MMPs consist of a pre-, pro- and catalytic domain and, apart from MMP-7, -23 and -26, also contain a hinge region of varying length connecting a hemopexin domain (Table 1).[191,192] All MMPs except MMP-23 have a conserved sequence around cysteine in the propeptide (PR**C**GXPD), which is also found in the ADAMs and ADAMTSs. The cystein in this motif maintains the latency of the MMP. Limited proteolysis of the pro-peptide, treatment with chaotropic agents or organomercurials (APMA: *p*-amino-phenyl-mercuric acetate is widely used in *in vitro* experiments) disrupts the cystein-zinc bond and actives the enzyme.[193] A furin recognition motif (RX(R/K)R) is present on the carboxy terminus of the cysteine switch motif in several MMPs, allowing intracellular activation by furin-like pro-protein convertases in the Golgi apparatus. Recently, furin mediated inactivation of MMP-2 was observed and it appears other MMPs previously not recognized as furin substrates may also be targets.[194] The catalytic domain contains a conserved active site sequence: **H**EXX**H**XXGXX**H** with three histidine residues depicted in bold binding the zinc atom. The conserved sequence is shared with all other members of the metzincin group. MMP molecules contain additional non-catalytic zinc and calcium ions, which are involved in stabilizing the tertiary structure of the enzyme.[195,196] In the catalytic domain of MMP-2 and -9 three fibronectin type II repeats are inserted which bind gelatin and collagen thus facilitating the breakdown of these substrates. The flexible O-glycosylated proline-rich linker of MMP-9 is exceptionally large and facilitates binding of the enzyme to TIMP and cargo transporters.[197] The hemopexin domain co-determines substrate specificity and affinity. It binds Tissue Inhibitor of Metalloproteinases (TIMP, natural inhibitors of

MMPs) and docking molecules on cell surface membranes, for instance integrins and CD44. The hemopexin domain is also involved in MMP di-/oligomerization and CD97 mediated MMP uptake and internal degradation.[198] The MT-MMPs are connected to the cell by a short hydrophobic transmembrane segment or alternatively are glycosylphoshatidylinositol (GPI) anchored, focusing the cell's proteolytic capacity. However, cleavage at the stem region by other MMPs or ADAMs may release an active ectodomain.[199,200] The cysteine array and immunoglobulin-like domain in MMP-23 are of unknown function. The structural organization of the MMPs is also observed in the ADAMs and ADAMTSs, but these enzymes lack the hemopexin domain and possess other C-terminal extensions instead.[186]

Matrix metalloproteinases - expression

The expression of MMPs is tightly controlled at the transcriptional, translational and/or secretory level. Pro-inflammatory cytokines, chemokines, growth factors and oxidants including IL-1α, IL-1β, IL-6, IL-12, TNF-α, bFGF, MCP-1 and manganese superoxide dismutase (Mn-SOD) generated H_2O_2 upregulate the expression of several members of the MMP family including but not limited to MMP-1, -2, -3, -9, -12 and/or -13 in a variety of cells encompassing fibroblasts, enterocytes, T cells, chondrocytes, osteoblasts, endothelium and/or macrophages, sometimes in a synergistic manner.[201-211] Anti-inflammatory (TGF-β), pleiotropic (IFN-γ) cytokines and steroid sex hormones (estradiol) may downregulate MMP, whereas other members of the MMP family (i.e., MMP-2) display a rather constitutive expression rate in distinct cell types.[212-215] Importantly, results for a given combination of cytokine and MMP are not only dependent on cell type but also at what time point in their development and under what (experimental) conditions these cells are studied. Expression is also regulated by cell contact with the surrounding matrix, neighboring cells and pathogens. For instance: collagen I is able to induce MMP-1 in migrating keratinocytes thus promoting its own degradation and ligation of the fibronectin receptor α4/5β1, CD40 and TLR-2 may induce MMP-9.[216-220] The extrinsic signals are relayed to the MMP promoter by one or more intracellular signaling pathways, including NFκβ, SMAD, STAT, MAPK kinase pathways and

are integrated at cis-acting elements in the promoter, resulting in altered mRNA transcription rate.[221] Promoter activity is also dependent on DNA and histone methylation, acetylation and/or phosphorylation status.[222,223] Also, single nucleotide polymorphisms may result in the loss or gain of suppressor/enhancer DNA elements, affecting mRNA transcription. For instance, the replacement of cytosine with thymidine at -1306 in the MMP-2 promoter disrupts an Sp1 binding site, resulting in significantly decreased promoter activity.[224] A single guanine insertion at -1607 of the MMP-1 promoter creates an Ets binding site, elevating the transcriptional level of MMP-1.[225] The 3'UTR may contain ARE elements binding Hu and KH type splicing regulatory protein (KSFP) proteins increasing or decreasing mRNA stability, respectively, a mechanism shared with TNF-α and other pro-inflammatory cytokines.[226] The 3'UTR of MMP-9 mRNA is involved in binding cytoplasmic nucleolin, promoting transport to polyribosomes and enhancing protein translation efficiency.[227] Neutrophils and eosinophils store MMP-8, -9 and/or MT6-MMP in secretory granules which are released upon stimulation with pro-inflammatory cytokines such as IL-8 and TNF-α.[228-230] MT-1 MMP is stored in trans-golgi network/endosomes and may be expressed on the cell surface within minutes following Concanavalin A treatment of HT1080 cells.[231] Vesicular trafficking from the Golgi apparatus to the plasma membrane is dependent on actin and tubulin polymerization and can be suppressed by exposure to hypoxia, with concomitant drastic reduction of MMP secretion in monocytes.[232]

Matrix metalloproteinases - activation, inhibition and degradation

Except for furin-like pro-protein convertase-activated MT-MMPs and MMP-11, all other MMPs are secreted as inactive zymogens. Limited proteolysis by plasmin, thrombin, trypsin and other proteinases removes part of the propeptide region, inducing a conformational change in the MMP molecule disrupting the bond between the protective cysteine and catalytic zinc residue. Autocatalysis subsequently removes the entire propeptide region after which the enzyme becomes fully active.[233] The cysteine switch dogma is challenged by observations

of mutant MMP-3 with the cysteine replaced by serine or histidine. These molecules retained latency and could be activated with APMA, results not consistent with cysteine as primary regulator of MMP latency.[234] The final MMP activity is dependent on the activating proteinase, i.e., MMP-3-activated MMP-1 displays a higher conversion rate of collagen substrate compared to plasmin-activated MMP-1.[235] Contact with substrate or even non-functional protease may induce conformational change and activation of MMP without loss of the pro-peptide.[236,237] Oxidative activation and inactivation may play an important role during inflammation.[238] MMP-2 can be activated as described above, but the activation pathway in a complex with MT-MMPs and TIMP-2 is believed to be the most important physiologically.[238] MMP stability can be enhanced by MMP- binding proteins. For instance, neutrophil-derived lipocalin protects MMP-9 and may worsen prognosis in breast and gastric cancer.[239,240] MMP activity is inhibited by the endogenous inhibitors TIMPs, but glycosylation status co-determines affinity for TIMP and activity of the MMP.[241] General antiproteinases such as α2-macroglobulin also inhibit MMPs and the resulting inhibitor-MMP complex is subsequently removed from the circulation by scavenger receptors on macrophages. MMPs undergo further autocatalysis, inactivating themselves. Uptake of soluble MMPs by cells is mediated by the LRP receptor followed by degradation in lysosomal vesicles.[198] MT-MMPs are internalized by dynamin-dependent endocytosis in clathrin-coated pits.[242,243]

Matrix metalloproteinases - substrate specificity

A whole array of structural matrix proteins including, but not limited to, collagen I-XI, proteoglycans, elastin, laminin, vitronectin, tenascin, entactin and fibronectin can be cleaved by one or more members of the MMP family. In addition, non-structural proteins such as cytokines, growth hormones and binding proteins (for instance: IL-8, TGF-β and IGFBP-3) are cleaved as well (Table 1).[244] MMPs may also act intracellularly, targeting myosin light chain and troponin in cardiac myocytes.[245,246] Although overlapping, every MMP is characterized by its own substrate specificity, determined by the size and shape of the substrate-binding pocket. For instance, the gelatinases preferentially cleave collagen IV and gelatin,

whereas MMP-1 and -8 preferentially convert collagen I and III. Importantly, most substrate specificities have been determined *in vitro* and remain to be confirmed *in vivo*. In addition, MMPs may act using a non-proteolytic mechanism. For example, binding of TIMP-2 to MMP-14 upregulates cell migration and proliferation by activation of ERK1/-2, a process mediated by the cytoplasmic tail of MMP-14 and not dependent on extracellular proteolytic activity.[247]

Tissue inhibitors of metalloproteinases

The four different TIMPs currently known in humans inhibit activated MMPs by forming non-covalent 1:1 stoichiometric complexes that are resistant to heat-denaturation and proteolytic degradation. TIMP-1 and TIMP-3 also inhibit members of the ADAM and/or ADAMTS family.[248] TIMPs also bind to the proform of MMP-2 and MMP-9, thus regulating the activation process of these MMP members. Different TIMPs have different MMP binding specificities, for instance, TIMP-1 binds preferentially (pro-) MMP-9 but not MMP-2 while TIMP-2 binds (pro-) MMP-2 and not MMP-9. TIMPs are expressed by a variety of cell types including fibroblasts, enterocytes and leucocytes. Expression may be regulated by several cytokines, growth factors, hormones, etc., or is constitutive instead, dependent on TIMP and cell type studied, similar to the regulation of MMP expression. Of note, cytokines (i.e., TGF-β) that repress MMP expression, may enhance levels of TIMP and collagen, promoting a fibrotic phenotype.[249] Expression is also dependent on DNA-methylation and histone-acetylation status.[250] Hypomethylation of the TIMP-1 promoter may result in TIMP-1 expression from the otherwise inactive X chromosome in females, resulting in an overall increase of TIMP-1 levels.[251,252] Conversely, hypermethylation of a TIMP-2 CpG island upstream of the transcription start site is associated with diminished TIMP-2 expression in cervical carcinoma.[253] TIMP-1 and -2 were originally identified as erythroid potentiating factors and it now appears TIMPs are more generally involved in cell growth and/or apoptosis. The TIMP effect may be anti-apoptotic through ligation of the CD63/integrin-β1 complex but also pro-apoptotic via inhibition of MMP-mediated degradation of cell death receptor.[254] Several mutations in the TIMP-3 gene introducing an extra cysteine-residue and promoting dimerization, are associated with Sorsby's fundus dystrophy

and probably other degenerative retinopathies.[255]

Matrix metalloproteinases and tissue inhibitors of metalloproteinases - expression in IBD

MMPs are involved in normal physiological processes where matrix turnover is important, such as wound healing, embryogenesis, angiogenesis, etc. They are also implicated in several disease pathologies such as arthritis, dental disease and cancer metastasis. In IBD, high levels of proinflammatory cytokines in inflamed ulcerated tissue are associated with aberrant expression of MMPs and also TIMPs, but the balance between MMPs and TIMPs appears shifted to a more proteolytic phenotype.[256-262] The increased MMP/TIMP ratio in IBD may result in excessive tissue breakdown and facilitate leucocyte extravasation and migration, although MMP-specific substrate cleavages in IBD mucosa have not been detected so far.[263] Excessive expression of MMPs may also enhance fibroblast trans migration, promoting fibrosis and stenosis, especially in CD.[264-266] Alternatively, the MMP over TIMP ratio may not be sufficiently enhanced to compensate for the increased collagen production by IBD fibroblasts, again resulting in fibrosis.[267] Targeting TIMP-1 with non-functional MMP-9 mutants inhibited liver fibrogenesis, in favor of the second hypothesis.[268] MMPs may also generate new epitopes by cleaving substrates, thus perpetuating the immune response.[269] In several IBD models, administration of synthetic MMP inhibitors improved disease course and DSS-induced colitis was attenuated significantly in MMP-9 deficient mice.[270-272] However, ablation of MMP-2 was observed to aggravate experimental colitis, demonstrating protective capacities of MMPs in IBD as well.[273]

Outline of the studies described in this thesis

Inflammatory bowel disease is of major concern in industrialized countries. Health and economical costs have prompted the initiation of studies aimed at revealing the epidemiology, etiology and pathogenesis of IBD. Both CD and UC are characterized by excessive tissue breakdown during inflammation. The matrix metalloproteinases are important in normal physiological and pathological tissue remodeling and repair processes, including IBD. A short overview of IBD and MMPs is given in **chapter 1**.

Several studies have documented on the altered expression of MMPs in IBD tissue. **Chapter 2** reports on the expression of MMP-2 and MMP-9 in IBD inflamed versus non-inflamed IBD and control intestinal mucosa. MMP levels were measured by enzyme linked immunosorbent assay (ELISA), zymography, activity assay and reverse transcription polymerase (RT-PCR) assay. The cellular localization of MMP expression was determined by immunohistochemistry.

Infliximab is administered to steroid refractory CD patients and targets TNF-α, disrupting proinflammatory communication and promoting apoptosis in leucocytes via reverse signaling. The effect of infliximab on MMP-1, -2, -3, -9 and TIMP-1, -2 protein expression is described in **chapter 3**. Intestinal explants were cultured *ex vivo* with/without (w/wo) infliximab and relative expression of MMP, TIMP, TNF-α was measured by ELISA, activity assay and/or RT-PCR. In addition, explants were cultured w/wo pokeweed mitogen (PWM), to study the expression profiles under inflammatory conditions.

Chapter 4 describes the MMP-2 and MMP-9 serological and mucosal expression profile after the administration of infliximab to CD patients with fistulizing or active disease. Whole blood cultures w/wo infliximab and/or lipopolysaccharide (LPS) were performed to study *in vitro* the contribution of TNF-α in the regulation of MMP-2 and MMP-9 mRNA and protein expression.

In **chapter 5** expression of MMP-1, -2, -3, -9 and TIMP-1, -2 as measured by ELISA and/or activity assays in a large collection of IBD and control intestinal mucosa and related to CD phenotype is reported. The net MMP activity was compared to MMP over TIMP ratio and correlated with myeloperoxidase (MPO) content.

Single nucleotide polymorphisms (SNP) in genes may affect mRNA transcription, stability and/or protein function, thus enhancing disease susceptibility and/or phenotype. **Chapter 6** documents the distribution of (functional) SNPs in the genes encoding MMP-1, -2, -3 and -9, TIMP-1, -2 and TNF-α in a large cohort of IBD patients versus control subjects. Results were correlated with protein expression and clinical course, i.e., development of fistulae, stenotic complications and organ involvement.

After surgical resection, CD patients often experience recurrence of disease. Numerous studies have attempted to identify causal factors and smoking has been established as a bad prognostic factor. In **chapter 7** the clinical course of a large cohort of fully documented CD patients was related to MMP and TIMP genotypes and protein levels in surgically resected intestinal mucosa. In addition, several clinical and demographic variables such as smoking habits, sex and age at resection were retrospectively collected and related to the clinical outcome as well. The different studies are finally compiled as a summarizing discussion in **chapter 8**.

Table 1 (next pages). MMPs and TIMPs, adapted from references.[191,192,244,274] Pre = prepeptide signal sequence, pro = propeptide, catalytic = catalytic domain, Zn = catalytic zinc, F = fibronectin type II repeat, Fu = furin pro-protein convertase cleavage site, TM = transmembrane region, cyt = cytoplasmic tail, GPI= glycosylphosphatidylinositol anchor, CA = cysteine array, Ig = immunoglobulin, V= vitronectin insert. Note: substrate specificities were determined *in vitro* and remain to be confirmed *in vivo*. MMPs may digest other substrates not mentioned in this overview. *Cellular expression is dependent on stimulation by cytokines, extracellular matrix, DNA-methylation and acetylation, oncogenic transformation, etc., and expression should not be viewed as limited to those cells or tissues mentioned. References indicated between brackets.

Table 1.

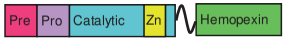
Subgroup	MMP	Trivial name	Chromo-somal location	Domain Organization	Subgroup substrate specificity	Individual MMP substrate specificiy	Cellular source*
Collagenases	MMP-1	Interstitial collagenase; collagenase 1	11q22-q23		Collagen I, II, III, VII, X, gelatin, entactin, aggrecan, tenascin, perlecan	IGFBP-2,3 Pro-MMP-1,2; pro-TNF-α, α1-antichymotrypsin, α1-proteinase inhibitor	Fibroblasts, keratinocytes, macrophages [275-277]
	MMP-8	Neutrophil collagenase; collagenase 2	11q21-q22			Pro-MMP-8, α1-proteinase inhibitor	Neutrophils, fibroblasts [278,279]
	MMP-13	Collagenase 3	11q22.3			Pro-MMP-9, 13; α1-antichymotrypsin	Chondrocytes, osteoblasts [280,281]
Gelatinases	MMP-2	Gelatinase A	16q13		Gelatin, elastin, fibronectin, collagen I/IV/V/VII/X/XI, laminin, aggrecan, vitronectin	Decorin, pro-TGF-β2, pro-IL-1β, MCP-3, IGFBP-3/5, pro-TNF-α, FGF-R1, pro-MMP-1, 2, 13	Fibroblasts, vascular smooth muscle cells, T lymphocytes [282-285]
	MMP-9	Gelatinase B	20q11.2-q13.1			Pro-TGF-β2, pro-IL-1β, IL-8, cell surface bound IL-2Ra, plasminogen, α1-proteinase inhibitor, pro-TNF-α	Neutrophils, monocytes/ macrophages, Fibroblasts [286-288]
Stromelysins	MMP-3	Stromelysin 1	11q23		Proteoglycans, laminin, fibronectin, gelatin, collagen III/IV/V/IX/X/XI, fibrin/fibrinogen, entactin, tenascin, vitronectin	Perlecan, decorin, pro-HB-EGF, pro-IL-1β, plasminogen, E-cadherin, IGFBP-3, α1-antichymotrypsin, α1-proteinase inhibitor, pro-MMP-1, 3, 7, 8, 9, 13, pro-TNF-α	Fibroblasts, chondrocytes, vascular smooth muscle cells, keratinocytes, macrophages and endothelial cells [289-294]
	MMP-10	Stromelysin 2	11q22.3-q23			Pro-MMP-1, 8, 10	Keratinocytes, monocytes, fibroblasts [294-296]

Table 1, continued.

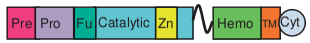
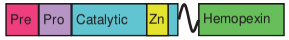
Subgroup	MMP	Trivial name	Chromo-somal location	Domain Organization	Subgroup substrate specificity	Individual MMP substrate specificiy	Cellular source*
Matrilysins	MMP-7	Matrilysin 1	11q21-q22			Proteoglycans, laminin, fibronectin, gelatin, collagen III, IV, V, IX, X, XI, fibrin/fibrinogen, entactin, tenascin, vitronectin, pro-α-defensin, decorin, cell surface bound FasL, β4-integrin, E-cadherin, plasminogen, pro-TNF-α, pro-MMP-2, 7	Epithelial cells [297,298]
	MMP-26	Matrilysin 2	11p15			Gelatin, collagen IV, fibronectin, fibrinogen, α1-proteinase inhibitor, MMP-9	Placenta, uterus, ovary, keratinocytes [299-301]
	MMP-11	Stromelysin 3	22q11.2			Laminin, fibronectin, aggrecan, α1- proteinase inhibitor, IGFBP-1	Fibroblasts, B-lymphocytes [295,302]
Membrane type MMPs -trans-membrane type	MMP-14	MT1-MMP	14q11-q12		Gelatin, fibronectin, vitronectin, collagen, aggrecan	Pro-MMP-2, 13; cell surface bound CD44, cell surface bound tissue transglutaminase (tTG)	Endothelial cells, hepatic stellate cells, fibroblasts [303-305]
	MMP-15	MT2-MMP	15q13-q21			TTG	Central Nervous system microglia [306]
	MMP-16	MT3-MMP	8q21			Pro-MMP-2, tTG	Chondrocytes [307]
	MMP-24	MT5-MMP	20q11.2			Pro-MMP-2	T-lymphocytes, fibroblasts [277,295]

Table 1, continued.

Subgroup	MMP	Trivial name	Chromo-somal location	Domain Organization	Subgroup substrate specificity	Individual MMP substrate specificiy	Cellular source*
Membrane type MMPs -GPI anchored	MMP-17	MT4-MMP	12q24.3			Gelatin, pro-MMP-2	Monocytes, B-lymphocytes [295]
	MMP-25	MT6-MMP	16p13.3			Gelatin, collagen IV, fibrin, fibronectin, laminin-1, pro-MMP-2	Monocytes, neutrophils [229,295]
Others	MMP-12	Macrophage elastase	11q22.2-q22.3			Elastin, fibronectin, fibrin, fibrinogen, laminin, proteoglycan, plasminogen	Macrophages [308]
	MMP-19	-	12q14			Gelatin, tenascin, fibronectin, collagen IV, laminin, entactin, fibrin/fibrinogen, aggrecan, COMP	Keratinocytes, brain microglia [309,310]
	MMP-20	Enamelysin	11q22.3			Amelogenin, aggrecan, COMP	Tooth enamel forming ameloblasts (epithelial origin) [311]
	MMP-21	-	?			Gelatin	Keratinocytes [312]
	MMP-23	CA-MMP	1p36.3			Gelatin	Reproductive organs [313]
	MMP-27	-	11q24				B-lymphocytes [295]
	MMP-28	Epilysin	17q21.1				Keratinocytes, intestine, T-lymphocytes [257,295,314]

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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

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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.

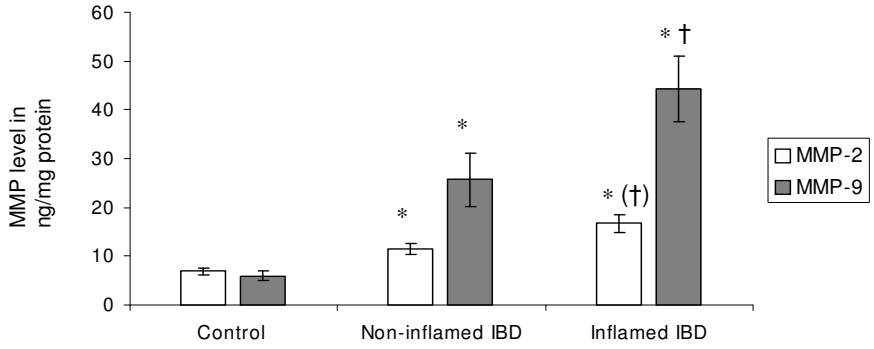

Figure 1. MMP-2 (open bars) and MMP-9 (grey bars) ELISA results in mean ± S.E.M. There was a highly significant difference between control and IBD tissues; the MMP levels increased with inflammation. $^{*}P \leq 0.005$ vs. controls;$^{(\dagger)}$ $P = 0.07$; $^{\dagger}P = 0.005$ vs. non-inflamed. IBD: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

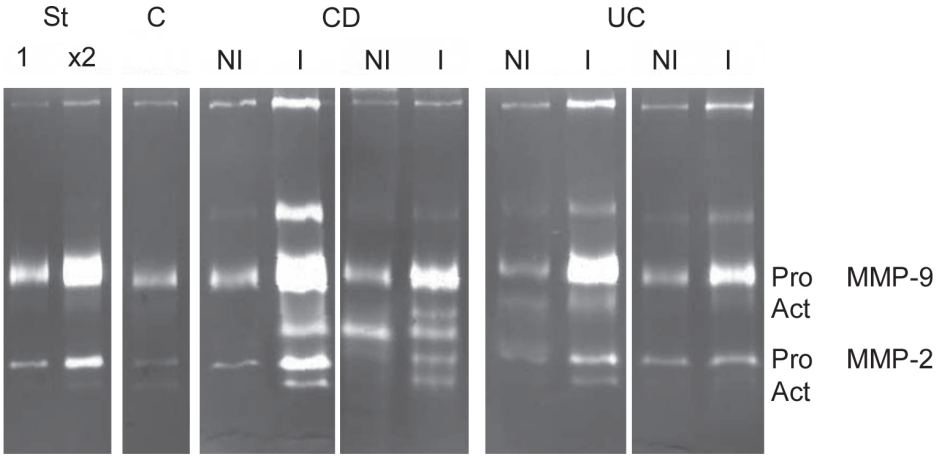

Figure 2. Example gelatin zymograms of non-inflamed and inflamed IBD tissue homogenates compared to control tissue and internal standard, identifying pro- and active MMP-2 and MMP-9. St: internal standard, C: control, CD: Crohn's disease, UC: ulcerative colitis, NI: non-inflamed, I: inflamed, Pro: pro-enzyme,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

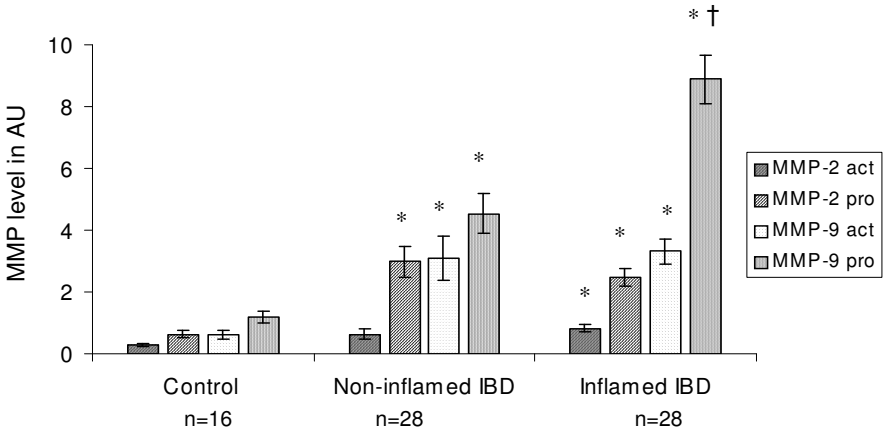

Figure 3. MMP-2 (hatched bars) and MMP-9 (dotted bars) zymography results in mean ± S.E.M. Both the active and pro-enzyme forms of MMP-2 and MMP-9 were increased in IBD tissues, only pro-MMP-9 increased further with inflammation. $^{*}P \leq 0.02$ vs. controls; †$P = 0.0005$ vs. non-inflamed, AU: arbitrary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

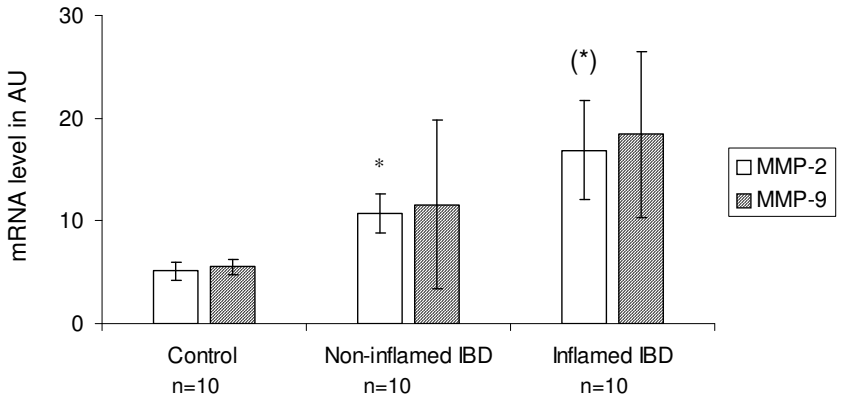

Figure 4. MMP-2 (open bars) and MMP-9 (hatched bars) RT-PCR results in mean ± S.E.M. Similar to the expression pattern of the proteins there was an increase in mRNA in IBD tissues, and in relation with inflammation, compared to that in control tissues. $^{(*)}P = 0.09$; $^{*}P = 0.03$ vs. controls. AU: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

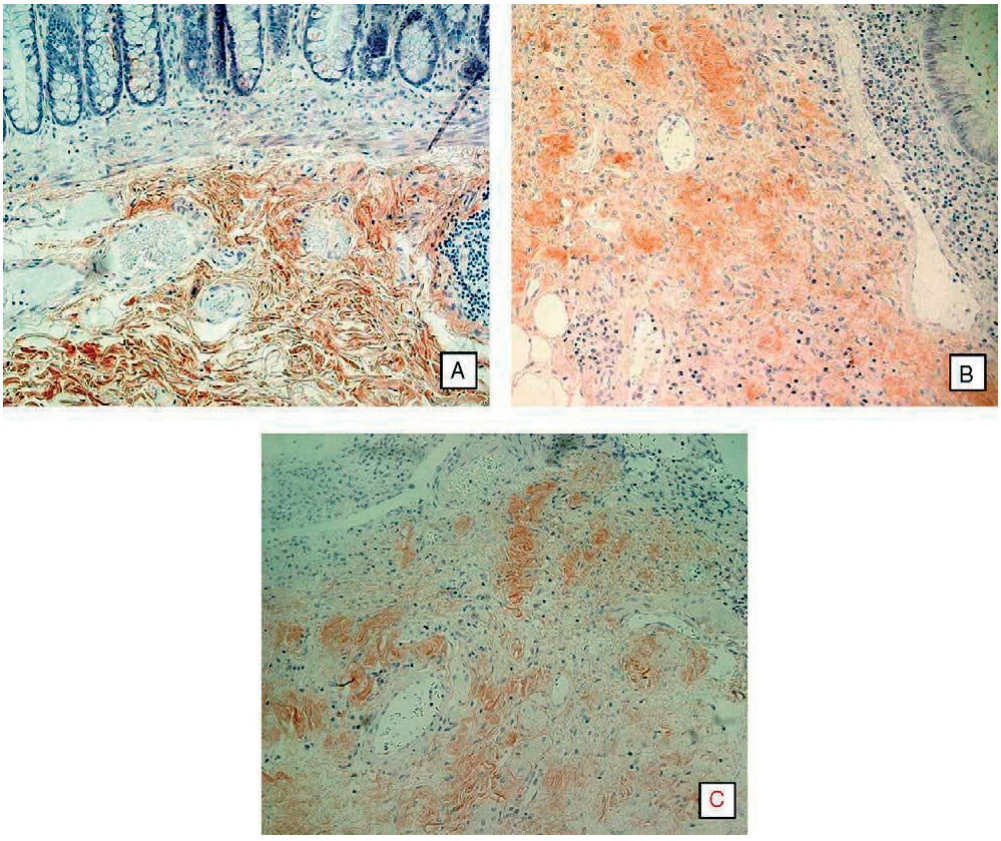

Figure 5. The prominent location of MMP-2 was in the submucosa, especially in controls (A, ×200); in inflamed tissues of CD and UC (B and C, ×200) MMP-2 was seen as a weak but more generalized positive reaction in the ECM.

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

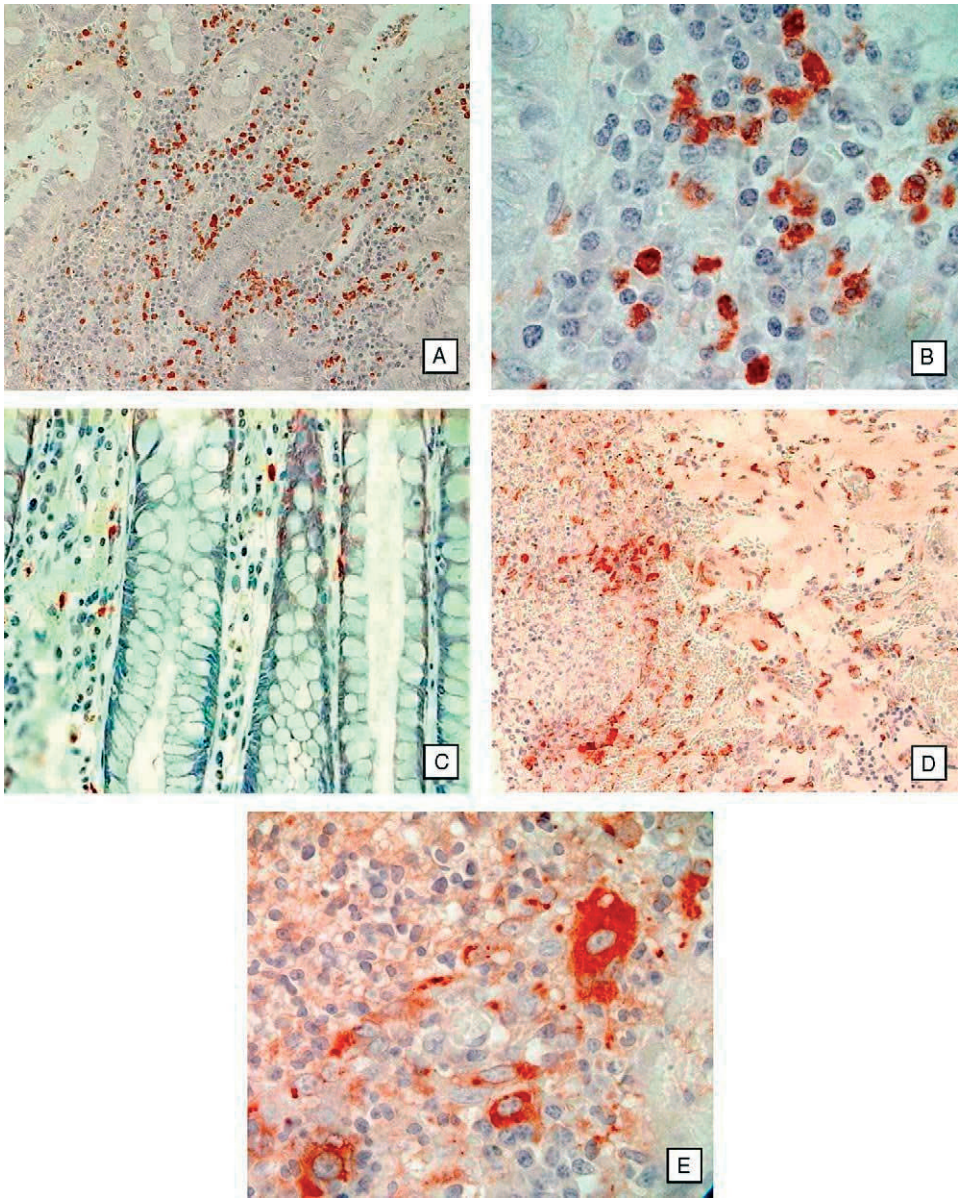

Figure 6. A pronounced positive staining for MMP-9 in leukocytes in inflamed areas of CD was found (A, ×200); neutrophils in inflamed tissue of CD were strongly positive for MMP-9 (B, ×1000); few leukocytes in control tissue were MMP-9 positive (C, ×400); MMP-9 in cells and ECM in UC inflamed area surrounding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

Acknowledgements

Q.G. was a recipient of a scholarship from the Chinese Scholarship Council. Part of the study was supported by a grant from the Gastrostart Foundation, The Netherlands. Dr. Roeland Hanemaaijer and Dr. Jan Verheijen from the Gaubius Laboratory TNO-PG, Leiden, The Netherlands are gratefully acknowledged for their technical assistance in the MMP analyses.

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Chapter 3

Effect of the Anti-Tumor Necrosis Factor-α Antibody Infliximab on the *ex vivo* Mucosal Matrix Metalloproteinase-Proteolytic Phenotype in Inflammatory Bowel Disease (IBD)

Short title: Infliximab and MMPs in IBD

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This Study was published in Inflamm Bowel Dis 2007;13:200-210

Abstract

Background/aims. Previous studies have shown an upregulation of matrix metalloproteinases (MMPs) in intestinal tissue of patients with inflammatory bowel disease (IBD) and significant clinical improvement after administration of the anti-TNF-α antibody infliximab. The aims of our study were to determine expression and secretion of MMP-1, -2, -3, -9, and their inhibitors TIMP-1, -2 by IBD versus control intestinal mucosa *ex vivo* and to assess the regulatory capacity by infliximab of the proteolytic phenotype.

Methods. Intestinal mucosal explants from 20 IBD and 15 control patients were cultured with or without infliximab and/or the T-cell activator pokeweed mitogen (PWM). Explants and culture supernatants were analyzed for MMPs, TIMPs, and TNF-α protein, activity and/or mRNA levels. All patients were genotyped for functional TNF-α, MMP, and TIMP single nucleotide polymorphism (SNP) loci.

Results. Expression of MMP and TIMP protein/activity in basal medium was higher in IBD versus control explants. Dependent on genotype at SNP loci, infliximab downregulated MMP-1, -3, and -9 relative to TIMP-1 and -2 and also decreased MMP-1 and -3 activities, while PWM enhanced these levels, partly counteracted again by infliximab. The expression of MMP-2 relative to TIMP did not change by treatment with infliximab and/or PWM.

Conclusions. The high expression of MMPs in patients with IBD suggests a role for these proteinases in the pathogenesis of this disease. Infliximab seems to induce a genotype-associated matrix protective phenotype, which may contribute to the observed therapeutic efficacy of this drug in IBD, particularly at the mucosal surface.

Introduction

Patients with Crohn's disease (CD) suffer from multifocal transmural inflammation potentially affecting the whole gastrointestinal lining, but most often localized in the ileocecal region, while patients with ulcerative colitis (UC) have a more superficial continuous mucosal inflammation, affecting the colon only. In both forms of inflammatory bowel disease (IBD), the sustained inflammation may cause severe tissue damage, translating into clinical complaints such as diarrhea, cramps, weight loss, blood iron deficiency, etc. When refractory to medical treatment or due to complications this may result in surgical resection of the affected bowel. Early studies have shown an upregulation of several cytokines in IBD and TNF-α seems to be pivotal. The TNF-α mRNA and/or protein expression was shown to be upregulated in blood,[1] intestinal mucosa,[2,3] stools,[4] and cultured intestinal biopsies.[5] Considering its pleiotropic proinflammatory nature, a chimeric monoclonal antihuman TNF-α antibody was engineered[6] and found to be beneficial to a large proportion of CD and UC patients refractory to standard treatment with steroids.[7-9] Matrix metalloproteinases (MMPs) constitute a family of diverse matrix degrading zinc- and calcium-activated neutral endoproteinases, some of which are under cytokine control and are implicated in a wide range of biological processes including angiogenesis, implantation, and cancer metastasis.[10] A marked upregulation of MMP-1, -2, -3, -7, -9,-10, -12, -13, and -14 mRNA and/or (active) protein levels has been demonstrated in the intestinal mucosa of IBD patients, shifting the balance between these proteinases and their natural inhibitors, i.e., tissue inhibitors of metalloproteinases (TIMPs),[11] toward a net proteolytic activity.[12-17] The enhanced expression of MMPs near ulcera in inflamed IBD intestinal tissue reinforces the potential pathogenic role of these proteinases. Also, in the rat trinitrobenzenesulfonic acid and human pokeweed mitogen (PWM)-activated fetal intestinal models of IBD the application of MMP inhibitors was associated with a decrease in inflammation score and/or tissue damage,[18-20] while in the murine dextran sodium sulfate model of UC inhibition of macrophage migration inhibitory factor was associated with reduction of inflammation, tissue damage, and diminished MMP-13 expression.[21] Based on these data, we speculated that infliximab might be of clinical benefit through an

inhibition of TNF-α-mediated increase in net MMP activity. We experimentally manipulated the concentration of bioavailable TNF-α in IBD mucosa *ex vivo* and studied the effects on the expression and/or activities of MMP-1, -2, -3, -9, and TIMP-1, -2. Recently, a number of single nucleotide polymorphisms (SNPs) in the genes coding for TNF-α, MMPs, and TIMPs have been described and several of these SNPs were shown to be functional and/or related to the pathogenesis of various diseases.[22-27] We therefore also examined the effects of these SNPs on TNF-α-regulated MMP and TIMP expression.

Patients and methods

Patients and tissue samples

Demographic and clinical data of the patients are depicted in Table 1. Diagnosis of CD or UC was based on standard clinical, endoscopic, radiologic, and histopathologic findings. Judged by the presence of ulcera, erythematous appearance, marked bowel wall thickening, and/or loss of circular folding, surgically resected IBD tissue was considered macroscopically normal or affected. The control group consisted of three patients with diverticular disease and 12 patients with neoplasia; normal tissue was collected at least 10 cm away from affected tissue. Most IBD but none of the control patients received immunosuppressive therapy prior to surgery.

Tissue culture

Resection specimens were obtained at the Pathology Department of the Leiden University Medical Center within 1 hour after surgical removal and transported in L-15 medium supplemented with penicillin and streptomycin to the laboratory of the Gastroenterology Department. After thorough washing in L-15, mucosa and submucosa were carefully separated from muscularis externa and serosa. Mucosal layers were cut in ≈ 3x3 mm preweighted explants and cultured per 10 parts in 6-well culture plates containing 3.5 mL CMRL-1066 basic medium, modified according to Autrup *et al*,[28] with the omission of cortisone. Culture plates were

Table 1. Demographic and clinical data of the IBD and control patients included in the explant cultures

	CD	UC	Control
No. of patients	11	9	15
Median age in years (range)	34 (16–54)	42 (24–75)	66 (41–83)
Male/female	4/7	4/5	8/7
Ileum/colon	5/16	1/10	1/15
Normal/affected tissue	6/15	1/10	16/0
No. of patients with medication			
— Mesalazine	8	4	-
— Steroids	9	7	-
— Azathioprine, cyclosporine	6	6	-
— Infliximab	1	0	-

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis.

positioned in a sealed box (Billups-Rothenberg, Del Mar, CA), which was subsequently filled with 95% O_2/ 5% CO_2 according to the manufacturer's instructions and placed on a rocking platform. Explants were cultured for 72 hours at 37 ^{0}C with replenishment of medium at 24 and 48 hours, and supernatants and explants were stored at -70 ^{0}C until subsequent analysis. Infliximab (Centocor, The Netherlands, final concentration 0.14 mg/mL corresponding to physiological plasma concentration in CD patients and completely neutralizing TNF-α biological activity[29]) and T/B cell activator pokeweed mitogen (PWM, Sigma, The Netherlands, final dilution 1:100 according to manufacturer's protocol) were added to parallel incubations.

Immunohistochemistry

Frozen explants were cut into 7-µm sections and fixed in formaldehyde and Carnoy's modified medium. After blocking of endogenous peroxidase and nonspecific binding by 0.3% hydrogen peroxide in methanol and normal goat serum, respectively, sections were incubated overnight with mouse antihuman cytokeratin 18 antibody (Santa Cruz Bio-technology, Santa Cruz, CA).

Subsequently, biotinylated goat antimouse antibody and streptavidin-HRP conjugate (DAKO, Glostrup, Denmark) were added. Conversion of amino-9-ethylcarbazole in the presence of hydrogen peroxide resulted in a clear red staining. Sections were lightly counterstained in Mayer's hematoxylin and mounted in Aquamount.

Protein determination

Tissue homogenates (25 mg/mL) were prepared in 0.1 M Tris-HCl, 0.1% Tween 80, pH 7.5, using a Potter device (B. Braun, Germany).[30] The TNF-α (Biosource, Camarillo, CA), MMP-2, -9 (in-house), TIMP-1,-2 (R&D Systems, Minneapolis, MN) protein levels in tissue homogenates and/or culture supernatants were measured in parallel with appropriate standards by highly specific enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions or as described previously.[31,32] Values of TNF-α obtained from infliximab containing samples were multiplied 5-fold to correct for 80% inhibition of measurement of TNF-α by this antibody.[33] The MMP ELISAs recognize latent, active, and TIMP-complexed forms with comparable high efficiencies. Levels of MMP-1 and -3 were measured by specific immunocapture bioactivity assays from Amersham Biosciences (Arlington Heights, IL).[34] Briefly, MMP was captured from samples by immobilized MMP-specific antibodies and incubated in buffer with/without *p*-aminophenyl mercuric acetate (APMA) to measure the total of APMA activatable and endogenously active MMP (MMPt) or active MMP only (MMPa), respectively. Subsequently, pro-urokinase modified to contain an MMP recognition cleavage site was added and the color of chromogenic substrate converted by MMP-activated urokinase was measured at 405 nm. Values are expressed in arbitrary units and show good linear correlation with sample MMP protein.

Semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR)

Intestinal mucosa in 4 mL guanidinium thiocyanate solution was homogenized using a Turrax device (IKA-Werke, Germany) and RNA was isolated as described previously.[35] Four micrograms of DNAse (Sigma)-treated RNA were reverse

transcribed with MMLV reverse transcriptase (Invitrogen, The Netherlands) in a final volume of 40 µL according to the manufacturer's protocol with only small modifications. Subsequently, cDNA was diluted 20–40,000-fold in a final volume of 25 µL and amplified with Red Taq DNA polymerase (Sigma) and target-specific primers using a thermal cycler from Biometra (Germany). The PCR protocol consisted of a 3' DNA denaturation step at 94 °C, 28–33 amplification cycles (30 sec at 94 °C, 45 sec at 56–60 °C and 1 min at 72 °C each) and a final extension step of 7 minutes at 72 °C. Oligonucleotide primers were published elsewhere[36] or designed using the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA) and are as follows (sense, antisense, 5' to 3'): GGGTCAGAAGGATTCCTATG and GGTCTCAAACATGATCTGGG (β-actin); GATATCGGGGCTTTGATGTA and TCCTTGGGGTATCCGTGTAG (MMP-1); AGGATCATTGGCTACACACC and AGCTGTCATAGGATGTGCCC (MMP-2); CGATGCAGCCATTTCTGATA and TGTGACAAGGTGCAAGCTAAG (MMP-3); CGCAGACATCGTCATCCAGT and GGATTGGCCTTGGAAGATGA (MMP-9); CCCCAGGGACCTCTCTCTAA and GGAAGACCCCTCCCAGATAG (TNF-α) resulting in 238, 398, 535, 486, 406, and 413 bp products. The amplified DNA was visualized on agarose gel by UV transillumination, scanned using SCION software (www.scioncorp.com), and quantified essentially as described by Murphy *et al*,[37] with β-actin normalized values representing expression in arbitrary units relative to a positive standard run in parallel. Appropriate RNA-MMLV and cDNA controls were run along and always found to be negative.

Single nucleotide polymorphism analysis

Genomic DNA reconstituted in Tris-EDTA buffer was routinely obtained using an adapted blood leukocyte DNA isolation protocol.[38] The genotype at (SNP) loci MMP-1 -1607 1G/2G, MMP-3 -1613 5T/6T, MMP-9 -1562 C/T, TIMP-1 +372 T/C, TIMP-2 +303 G/A, and TNF-α -308 G/A were determined by restriction fragment length polymorphism (RFLP) analysis. Primers, restriction enzymes, and gel restriction fragment pattern corresponding to genotype are specified in Table 2. The SNP at MMP-2 -1306 C/T was analyzed by tetra primer amplification refractory mutational system (ARMS) PCR, the principles of which are described elsewhere.[39]

Table 2. Specification of primers and restriction enzymes used for analysis of SNPs in MMP, TIMP, and TNF-α genes with size of PCR products on agarose gel and inferred genotype.

Gene/SNP	Forward and reverse primers	Restriction Enzyme	Products (bp)/genotype
MMP-1	GAAATTGTAGTTAAATCCTTAGAAAG	EcoN I	120;120+99+21;99+21
–1607 1G/2G	TATGGATTCCTGTTTTCTTTCTGC		1G1G;1G2G;2G2G
MMP-2	ACCAGACAAGCCTGAACTTGTCTGA		542+379;542+379+211;
–1306 C/T	TGTGACAACCGTCTCTGAGGAATG		542+211 CC;CT;TT
	ATATTCCCCACCCAGCACGCT		
	GCTGAGACCTGAAGAGCTAAAGAGTTG		
MMP-3	CTTCCTGGAATTCACATCACTGCCACCACT	Tth111 I	130;130+97+32;
–1613 5T/6T	GGTTCTCCATTCCTTTGATGGGGGGAAAGA		97+32 6T6T;6T5T;5T5T
MMP-9	ATGGCTCATGCCCGTAATC	SpH I	352;352+208+144;
–1562 C/T	TCACCTTCTTCAAAGCCCTATT		208+144 CC;CT;TT
TIMP-1	GCACATCACTACCTGCAGTC	BssS I	175;175+155+20;
+372 T/C	GAAACAAGCCCACGATTTAG	155+20	TT or T;CT;CC or C
TIMP-2	CCTCCTCGGCAGTGTGTG	TspR I	12+16;112+97+24+16;
+303 G/A	TAGGAACAGCCCCACTTCTG		97+24+16 GG;GA;AA
TNF-α	GAGGCAATAGGTTTTGAGGGCCAT	Nco I	126+21;147+126+21;
–308 G/A	GGGACACACAAGCATCAAG		147 GG;GA;AA

SNP, single nucleotide polymorphism; PCR, polymerase chain reaction. Analysis of SNP MMP-2 –1306 was performed by tetra primer ARMS PCR which involves four oligonucleotide primers but no restriction enzyme. Primers and restriction enzymes for the analysis of SNPs at MMP-1, MMP-3, TIMP-1 and TNF-α loci are from references 55, 56, 57, and 58, respectively.

Analysis of SNP MMP-2 -1306 was performed by tetra primer ARMS PCR, which involves four oligonucleotide primers but no restriction enzyme. Primers and restriction enzymes for the analysis of SNPs at MMP-1, MMP-3, TIMP-1, and TNF-α loci are from previous publications,[55-58] respectively.

Statistical analysis

At the protein level, data are presented as means ± SEM and statistical significance of differences between groups was assessed by (paired) Student's *t*-test. At the mRNA level, data are presented as median (range) and statistical analysis was performed by Mann–Whitney *U*- and Wilcoxon signed ranks test. Correlations were calculated by Spearman's ranks test; all *P*-values are two-tailed and tests were conducted using the SPSS statistical software, v. 11.0 (Chicago, IL).

Results

Validation of culture

After 72 hours of culture in basal medium, lamina propria, muscularis mucosae, and submucosa were still clearly identifiable. Mucosa contained considerable numbers of crypts and lamina propria was covered by large stretches of cytokeratin-18-expressing columnar epithelial cells, with interindividual variation depending on starting tissue. Occasional mucosal edema was noted (Fig. 1). Addition of PWM caused significant time-dependent deterioration of tissue and after 72 hours crypts and epithelial coverage were no more present. In the lamina propria, condensed nuclei heavily stained with eosin were frequently observed at 72 hours, most likely representing activated lymphocytes. Addition of infliximab to basal medium or next to PWM did not alter explant morphological appearance. Replenishment of medium at 24 and 48 hours more closely maintained physiological pH as judged by medium color but did not improve explant morphology.

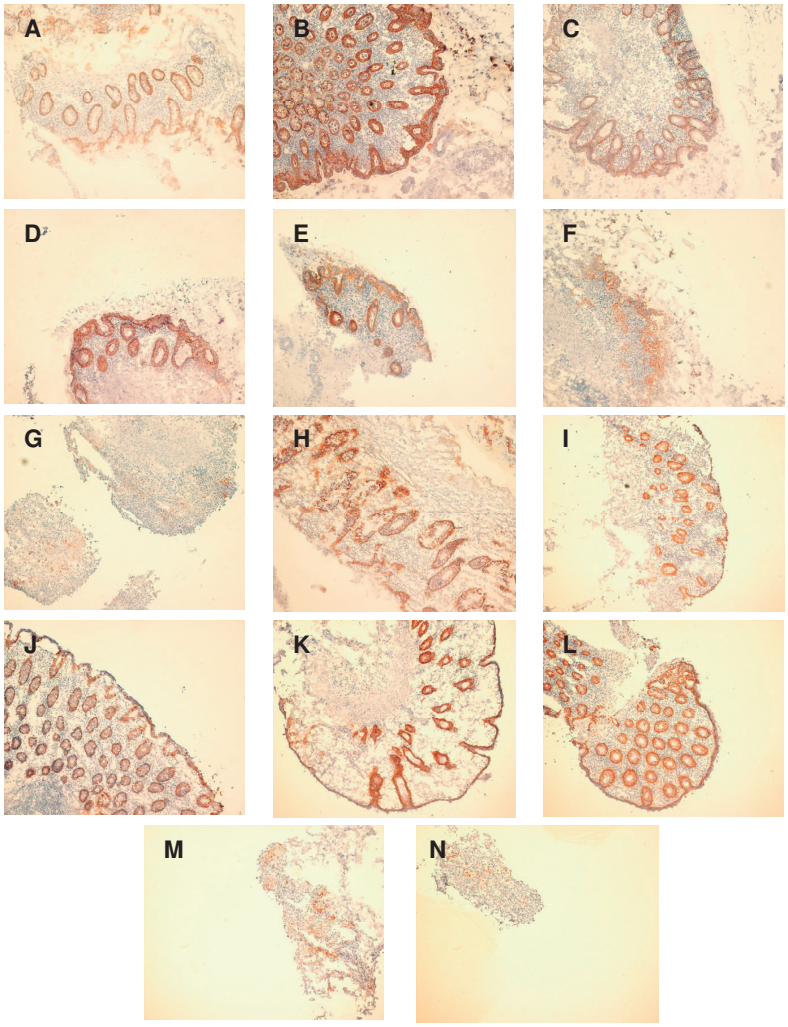
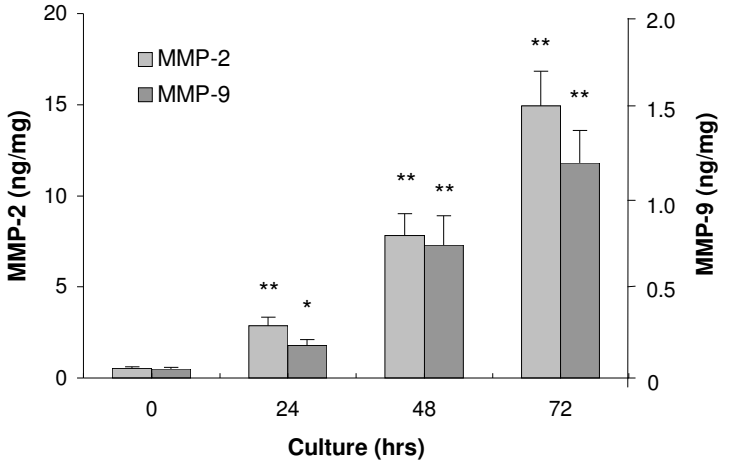


Figure 1. Immunohistochemical staining of cultured intestinal explants. Preculture tissue of a control patient (A) and after 24, 48, and 72 hours in basal medium (B–D, respectively). Note the good preservation of morphology and presence of red-colored cytokeratin-18-expressing epithelial cells. After incubation with PWM for 24 hours, tissue was still well preserved (E). However, prolonged incubation resulted in a gradual deterioration at 48 and 72 hours (F versus G). Fibrotic (H,I) and normal-appearing tissue (J–N) from a CD patient. After 72 hours culture in basal medium, morphology is well preserved (I, K) compared with preculture tissue (H, J). Incubation with PWM resulted in severe tissue destruction at 72 hours (M) and infliximab did not improve morphology, either next to PWM treatment (N) or added to basal medium (L). Similar results were obtained for (affected) CD, UC, and controls.

A)



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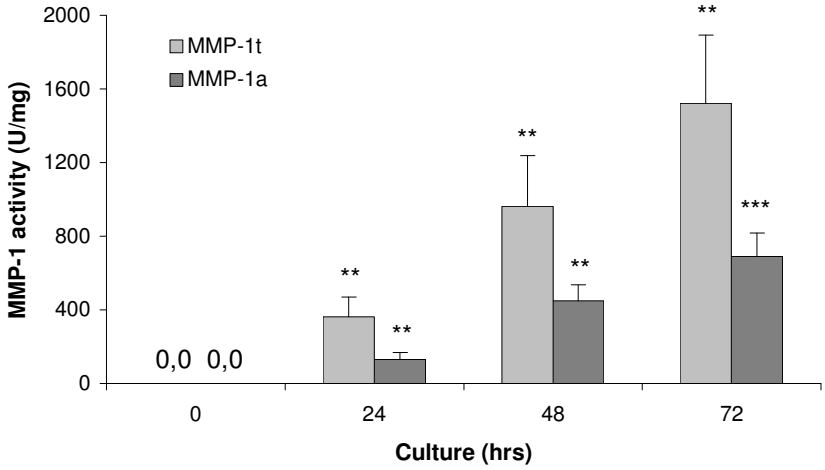
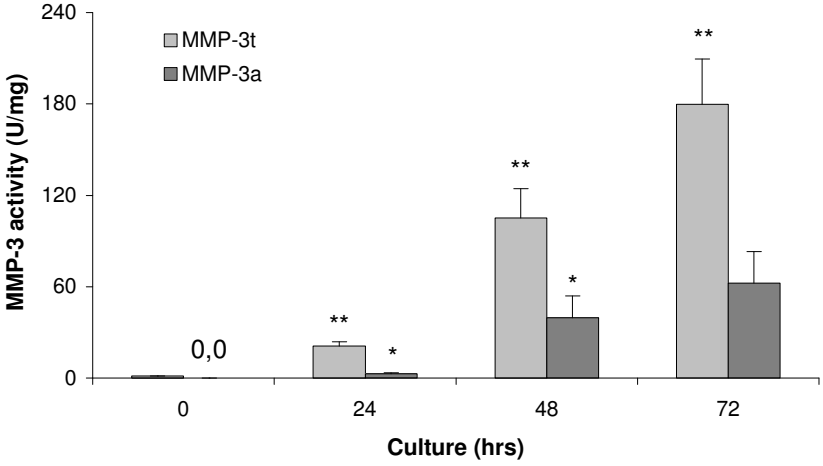


Figure 2. A–E: Basal protein expression/secretion by control intestinal explants. Preculture tissue homogenates (0) and supernatants at indicated times were measured. A: MMP protein in ng MMP/mg preincubation explant weight (mean ± SEM), $n = 10 –15$. $^{**}P < 0.01$, $^{*}P < 0.05$ versus level 24 hours earlier. B: MMP-1 activity in U MMP/mg preincubation explant weight (mean ± SEM) $n = 9 –10$. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus level 24 hours earlier.

C)



D)

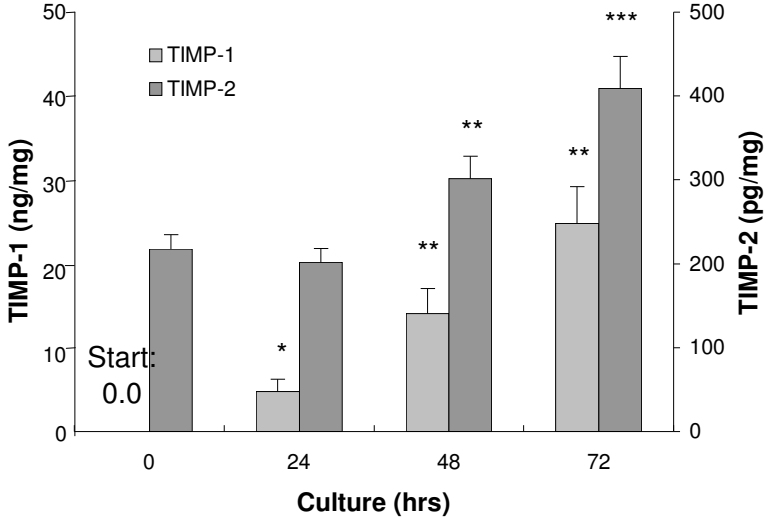


Figure 2 continued. C: MMP-3 activity in U MMP/mg preincubation explant weight (mean ± SEM) $n = 6$. $^{**}P < 0.01$, $^{*}P < 0.05$ versus level 24 hours earlier. D: TIMP protein in ng or pg TIMP/mg preincubation explant weight (mean ± SEM), $n = 6$. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ versus level 24 hours earlier.

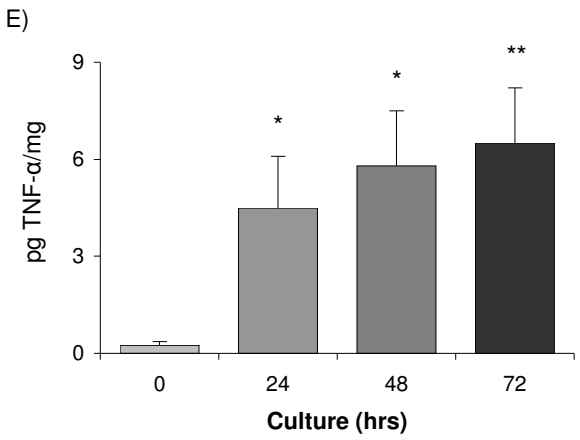


Figure 2 continued. E: TNF-α protein in pg TNF-α/mg pre-incubation explant weight (mean ± SEM), n = 9 –15. $^{**}P < 0.01$, $^{*}P < 0.05$ versus level 24 hours earlier.

Production of MMPs

During culture significant amounts of MMPs were continuously secreted into the medium (Fig. 2A,B), with a substantial fraction of MMP-1, -3 (30–50%) and the gelatinases (MMP-2: 15–55%, MMP-9: > 75%, data not shown) in the active form, as determined by BIA and gelatin zymography, respectively. Before culture, IBD explants already contained more MMP-2 (2-fold, P = 0.005), MMP-3t (3-fold, NS), and MMP-9 (10-fold, P = 0.002) compared to control tissue, while preculture MMP-1 and MMP-3a levels were below the detection limit of our BIA assay. After 72 hours MMP secretion in culture medium with/without infliximab and/or PWM by IBD explants was also significantly higher (Figs. 3, 4). Within the IBD group, UC compared to CD explants expressed higher MMP levels, i.e., basal MMP-1t: 44.6 ± 15.1 versus 27.6 ± 7.4 (10^3) U/mg, MMP-2: 27.3 ± 3.1 versus 24.4 ± 2.4 ng/mg, MMP-3t: 7.2 ± 2.4 versus 4.3 ± 1.0 (10^2) U/mg, and MMP-9: 3.1± 0.7 versus 2.2 ± 0.5 ng/mg at 72 hours, although not statistically significant. Addition of PWM resulted in opposite MMP-responses: MMP-2 level was downregulated about 50% compared to basal supernatants, whereas MMP-1, -3, and -9 levels were upregulated up to 40%. Incubation with infliximab also revealed divergent regulation pathways: basal and PWM immunostimulated MMP-2 secretion were not affected, but secretion of MMP-1, -3, and -9 was significantly downregulated by up to 40%, irrespective of tissue group. Remarkably, macroscopic disease had no major effect on MMP-2 and -9 production (e.g., basal MMP-9 secretion by affected

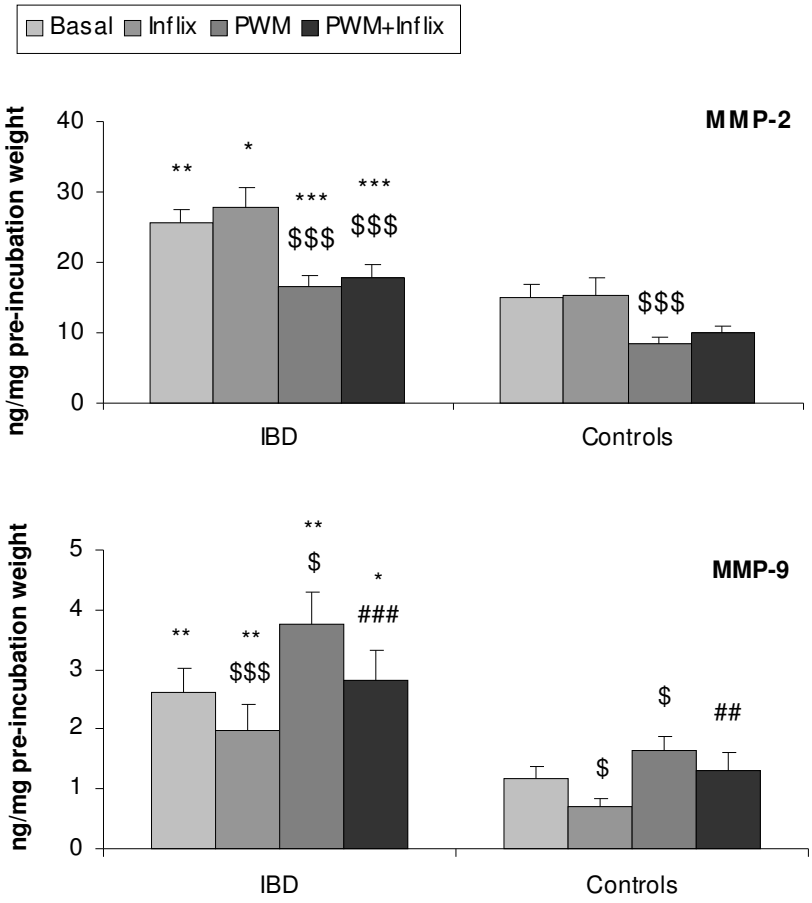


Figure 3. Cumulative MMP protein secretion by cultured intestinal explants at 72 hours (in ng MMP/mg preincubation explant weight, mean ± SEM). Explants from 15-20 IBD and 7-15 control patients were cultured w/wo infliximab and/or PWM. ***$P < 0.001$, **$P<0.01$, *$P<0.05$ versus corresponding explants from controls. $$$$P < 0.001$, $$$P < 0.01$, $ $P < 0.05$ versus basal incubation within same tissue group; ###$P < 0.001$, ##$P < 0.01$ versus PWM incubation within same tissue group.

IBD tissue was 1.9 ± 0.7 compared to 1.6 ± 0.5 ng/mg for normal IBD tissue, $n = 5$, paired *t*-test, $P = 0.4$; in case of MMP-1 and -3 only inflamed tissue was analyzed). Also, tissue location, i.e., ileum versus colon, had no effect (data not shown). Subsets of inflamed IBD and control tissues were also analyzed for mRNA expression. Preculture MMP mRNA levels were very low but higher in CD and UC

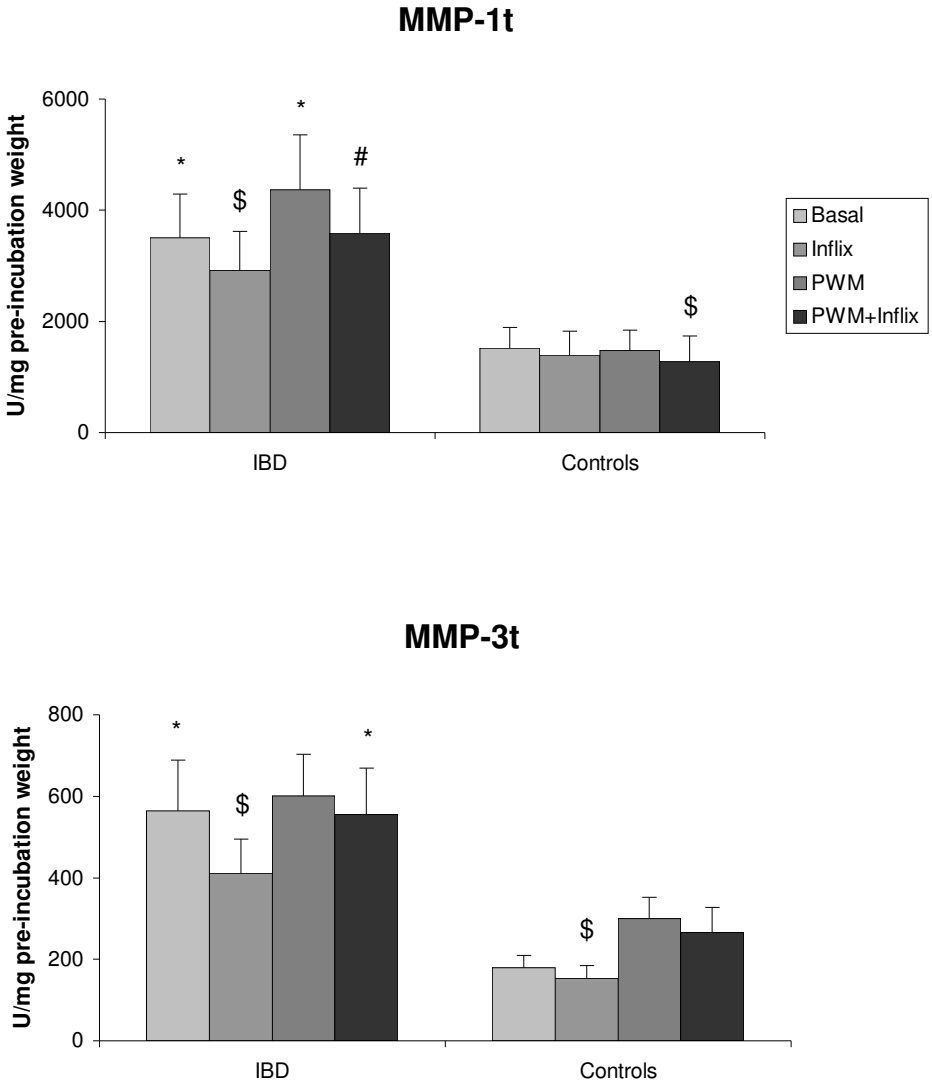


Figure 4-A. Cumulative MMP-t activity secretion (in U MMP/mg preincubation explant weight, mean ± SEM) by cultured inflamed IBD and control explants (MMP-1: n = 14–16 and 6–9; MMP-3: n = 13 versus 6, respectively) after 72 hours. **P < 0.01, *P <0.05 compared to corresponding control incubation, $$$P$ < 0.01, $$P$ < 0.05 versus basal incubation within same group, ##P <0.01, #P < 0.05 versus PWM incubation within same group. MMP activity was measured after preincubation with APMA.

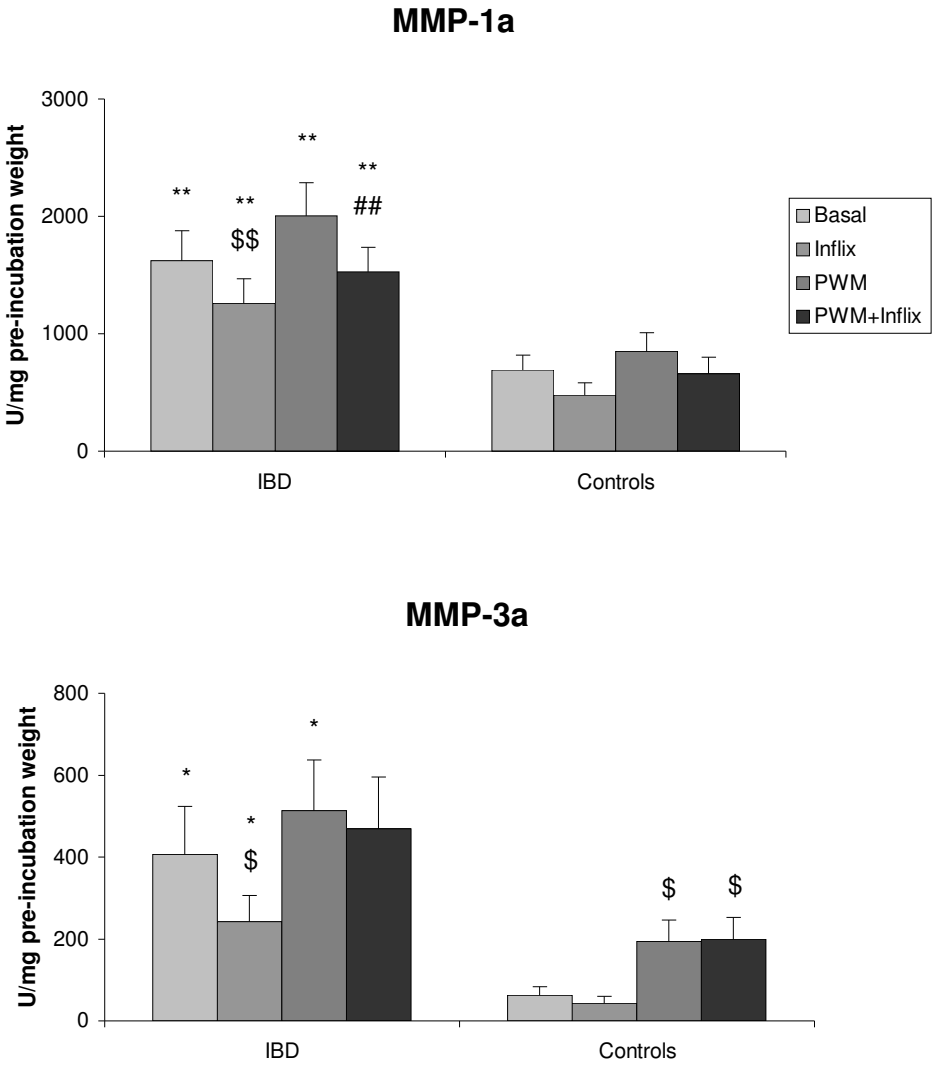


Figure 4-B. Cumulative MMP-a activity secretion (in U MMP/mg preincubation explant weight, mean ± SEM) by cultured inflamed IBD and control explants (MMP-1: n = 14–16 and 6–9; MMP-3: n = 13 versus 6, respectively) after 72 hours. $^{**}P < 0.01$, $^{*}P < 0.05$ compared to corresponding control incubation, $\$\$P < 0.01$, $\$P < 0.05$ versus basal incubation within same group, $\#\#P < 0.01$, $\#P < 0.05$ versus PWM incubation within same group. MMP activity was measured after preincubation without APMA.

compared to controls, and significant induction was observed during culture (MMP-2, -9: 4–16-fold and MMP-1, -3: 100–500-fold, see also Table X online supplementary material). Opposite to the protein, CD, UC, and control MMP mRNA levels at 72 hours appeared similar in corresponding incubations, although some differences were observed. Infliximab downregulated basal MMP mRNA expression, particularly MMP-1 and -3 in CD and controls but not UC, and when the results from CD and control tissue were combined, this was highly statistically significant (median percent change (range): - 40.9 (- 74.1 to 89.9, $P < 0.05$) and - 51.0 (- 78.8 to 37.4, $P < 0.01$), MMP-1 and -3, respectively). Incubation with PWM upregulated MMP mRNA expression 1.5–12-fold but concurrent incubation with infliximab could not prevent this increase.

Expression of TIMP-1 and TIMP-2

Affected IBD and normal control tissues were also analyzed for TIMP secretion. Both TIMPs were continuously produced and secreted throughout culture (Fig. 2C), with part of the TIMP-2 secretion reflecting release from the large protein pool already present in starting tissue. Before culture, IBD and control explants ($n = 13$ versus 6) contained similar amounts of TIMP (60 ± 50 versus 0 ± 0 pg/mg TIMP-1 and 267.2 ± 20.4 versus 217.5 ± 17.5 pg/mg TIMP-2, both NS) but 72 hours basal secretion was higher by IBD explants, especially in the case of TIMP-2 (Fig. 5). Within IBD, UC compared to CD explants expressed somewhat more TIMP, i.e., basal TIMP-1, -2 at 72 hours 48.0 ± 9.2 versus 28.9 ± 5.6 ng/mg and 7.5 ± 0.7 versus 6.2 ± 0.3 (10^2) pg/mg, P = NS, respectively. Addition of infliximab and/or PWM decreased TIMP protein secretion in all groups, but did not affect relative protein levels between corresponding incubations of IBD versus control tissue.

Production of MMP relative to TIMP

The effect of treatment on the weight ratio between MMP and TIMP is shown in Table 3. The MMP-2/TIMP ratios remained relatively stable. However, addition of infliximab to basal medium decreased the weight ratio between MMP-1t, MMP-3t, and MMP-9 versus TIMP-1 and/or TIMP-2 in CD, UC, and controls and when all groups were combined this effect was highly statistically significant. Apparently,

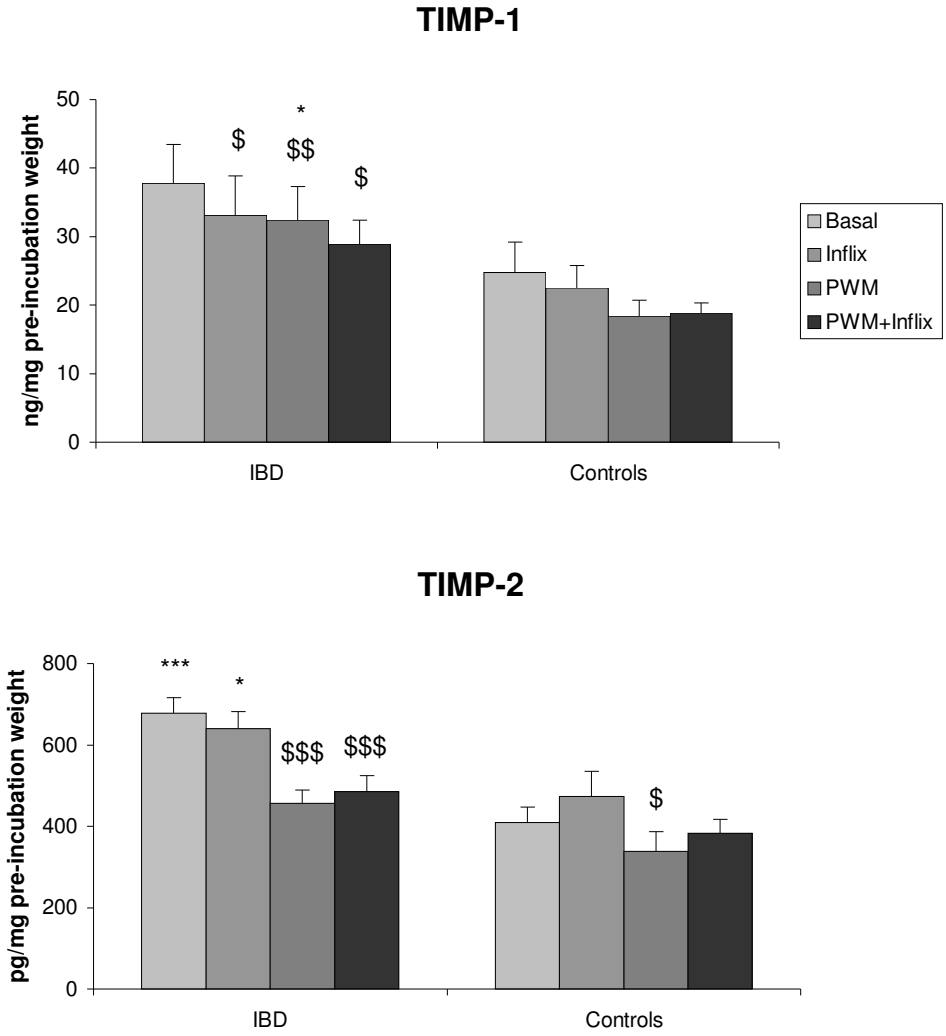


Figure 5. Cumulative TIMP protein secretion (in ng or pg TIMP/mg preincubation explant weight, mean ± SEM) by cultured intestinal inflamed IBD and control explants (n = 13 and 6, respectively) after 72 hours. ***P < 0.001, *P < 0.05 compared to corresponding control explant cultures, $$$$P$ < 0.001, $$$P$ < 0.01, $$P$ < 0.05 versus basal incubation within same group.

Table 3. Effect of incubation with infliximab and PWM on secretion of MMP relative to TIMP protein (mean ± SEM) by combined cultured inflamed CD, UC, and control intestinal explants (n = 13 and 6, respectively)

Ratio	Basal	Infliximab	PWM	PWM + infliximab
MMP-2/TIMP-1	0.8 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
MMP-2/TIMP-2	37.1 ± 1.9	38.6 ± 2.7	36.5 ± 3.1	36.2 ± 2.7
MMP-9/TIMP-1 (x10^3)	55.5 ± 8.9	42.0 ± 8.0**	121.9 ± 15.0**	80.2 ± 11.2*$^{\#\#}$
MMP-9/TIMP-2	3.4 ± 0.7	2.4 ± 0.6**	7.7 ± 1.0***	4.6 ± 0.7*$^{\#\#\#}$
MMP-1t/TIMP-1	93.2 ± 15.3	87.8 ± 14.0	127.8 ± 22.1**	110.0 ±18.4*
MMP-1t/TIMP-2	4.9 ± 0.9	4.1± 0.8**	7.5 ± 1.6**	5.8 ± 1.1*$^{\#}$
MMP-3t/TIMP-1	12.4 ± 1.4	11.2 ± 1.3	19.7 ± 2.1**	17.7 ± 2.1**
MMP-3t/TIMP-2	0.7 ± 0.1	0.5 ± 0.1*	1.2 ± 0.2***	1.0 ± 0.2$^{\#}$

CD, Crohn's disease; UC, ulcerative colitis; PWM, pokeweed mitogen. MMP-2 and MMP-9 versus TIMP reflect actual weight ratios, MMP-1 and MMP-3 versus TIMP values are in arbitrary units. ***P <0.001, **P < 0.01, *P < 0.05 versus basal incubation, $^{\#\#\#}P$ < 0.001, $^{\#\#}P$ < 0.01, $^{\#}P$ < 0.05 versus PWM.

levels of these MMPs are more dramatically reduced than either TIMP-1 or TIMP-2 when infliximab is added. Conversely, incubation with PWM increased these MMP/TIMP ratios toward a more proteolytic phenotype, and infliximab was able to partially inhibit these increases, although not fully back to control levels. The MMP/TIMP ratios in corresponding incubations from CD and UC compared to controls were higher, but statistical significance was not reached.

Production of TNF-α and relation to MMP/TIMP Expression

Explants also continuously secreted TNF-α into the medium, although peak production was observed during the first 24 hours (Fig. 2D). Secretion was paralleled by a concurrent increase of TNF-α in tissue homogenates (data not shown). Before culture, CD and UC explants contained similar high TNF-α levels compared to controls (0.80 ± 0.18 versus 0.25 ± 0.11 pg/mg, P < 0.05, n = 17 IBD versus 15 controls), and they had secreted more TNF-α , although not statistically significant, after PWM stimulation at 72 hours (Fig. 6). Incubation with infliximab only marginally affected basal TNF-α secretion. PWM, however, increased TNF-α

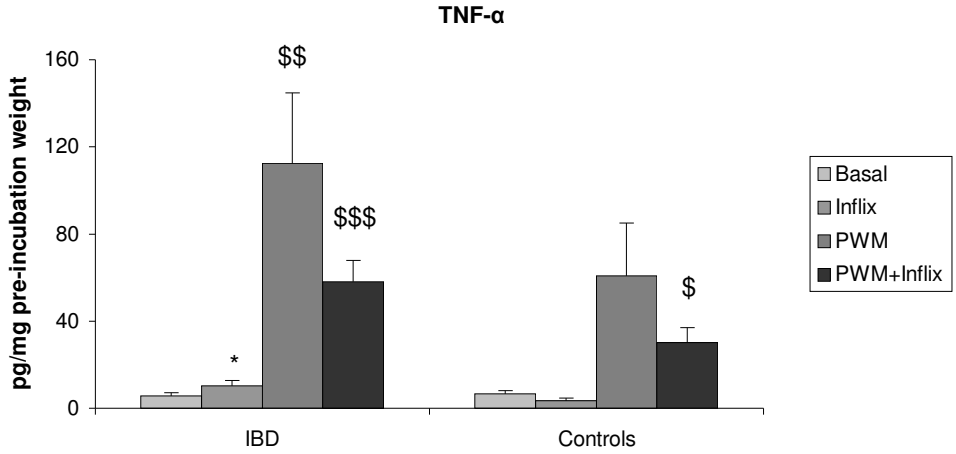


Figure 6. TNF-α protein secretion by intestinal inflamed IBD (n = 12–13) and control (n = 6–9) explants at 72 hours, cultured w/wo infliximab and/or PWM. Mean ± SEM in pg TNF-α /mg preincubation weights are shown. The TNF-α levels determined in medium supplemented with infliximab were multiplied 5-fold to correct for 80% inhibition of the TNF-α ELISA results by infliximab. *P < 0.05 versus control, \$\$\$$P$ < 0.001, \$\$$P$ < 0.01, \$$P$ < 0.05 versus basal incubation within same group.

levels 8–12-fold and infliximab was able to partially prevent this increase, although again not statistically significant. These results were essentially the same at the mRNA level (data not shown). There was a strong positive correlation of 72 hours basal TNF-α levels with MMP-3 total activity, MMP-9 and TIMP-1 protein secretion ($0.58 < r < 0.74$, $P < 0.05$, all groups combined, n = 18 –22). Importantly, basal TNF-α was also positively correlated with MMP-9/TIMP-1, MMP-9/TIMP-2, and MMP-3t/TIMP-2 ($0.67 < r < 0.85$, $P < 0.01$, n = 18), whereas a strong negative correlation was observed versus MMP-2/TIMP-1 (r = - 0.71, $P < 0.01$). All correlations were lost when explants were treated with PWM. Finally, MMP-3 is known to be a physiological activator of MMP-1. In our experiments, active MMP-3 and active MMP-1 were highly correlated independent of incubation with PWM and/or infliximab (e.g., basal level: r = 0.74, $P < 0.001$, n = 19).

Genotype at SNP loci and effect on protein and mRNA expression

Using RFLP or tetra primer ARMS PCR, relevant SNP loci in the DNA of IBD patients and controls were genotyped. Explants with at least one 2G, 5T, or A allele at MMP-1 -1607, MMP-3 -1613, or TNF-α -308, respectively, were shown to express more corresponding protein in medium with/without PWM and/or infliximab compared to explants with alternative allelic composition (Table 4), although not statistically significant. Allelic composition often did not affect the response of explants to infliximab or PWM; for instance, PWM significantly decreased MMP-2 protein expression in explants with the CC as well as in explants with the CT or TT genotype (CC basal compared to PWM: 20.1 ± 2.3 versus 12.4 ± 1.5 and CT + TT: 22.4 ± 2.2 versus 13.5 ± 2.0 ng/mg, both $P < 0.001$). However, in explants with the CC but not CT or TT genotype at MMP-9 -1562 infliximab significantly downregulated MMP-9 protein levels, while PWM caused an upregulation. Also, only in explants with at least one 2G or 5T allele at the MMP-1 -1607 versus MMP-3 -1613 locus, infliximab decreased total MMP-1 and MMP-3 secretion, respectively. At the mRNA level, explants with the CC genotype at MMP-2 -1306 expressed more MMP-2 than explants with the CT or TT genotype, although not statistically significant, and only explants with the 6T6T genotype at MMP-3 -1613 responded to incubation with infliximab by decreasing MMP-3 production ($P < 0.05$, see also Table XX online supplementary material).

Discussion

We have shown a generalized increased *ex vivo* expression of MMP-1, -2, -3, -9, and TIMP-1, -2 protein by explants from CD and UC versus control patients. The enhanced production of MMPs and TIMPs by IBD tissue corresponds to results from previous studies concerning expression levels of these markers *in vivo*[12-17] and could contribute to the tissue damage seen in IBD. In our experiments we observed that UC compared to CD explants expressed more MMP and TIMP, perhaps reflecting the more severe inflammation of the starting tissue of the former as measured by myeloperoxidase content (16.4 ± 1.4 versus 11.4 ± 1.1 U/g, $P < 0.05$). The protein level of MMP-2 dropped nearly 2-fold when the explants were activated by PWM, an inducer of the *in vitro* inflammatory process, while

Table 4. Genotype at selected SNP loci and effect on MMP, TIMP and TNF-α protein expression (mean ± SEM) by combined IBD and control explants

Protein	Genotype	No. Patients	Start	Basal	Infliximab	PWM	PWM + Infliximab
MMP-2 (ng/mg)	CC	14-21	0.7 ± 0.1†	20.1 ± 2.3	24.1 ± 3.6	12.4 ± 1.5***	15.1 ± 2.0**
	CT or TT	9-12	1.1 ± 0.2	22.4 ± 2.2	22.8 ± 3.0	13.5 ± 2.0***	15.2 ± 1.9*
MMP-9 (ng/mg)	CC	18-25	0.3 ± 0.1	2.0 ± 0.3	1.5 ± 0.3***	2.9 ± 0.4**	2.2 ± 0.4$^{\#\#\#}$
	CT or TT	5-8	0.5 ± 0.3	2.0 ± 0.8	1.9 ± 1.4	2.6 ± 0.7	2.6 ± 0.9$^{\#}$
MMP-1t 10^3 U/mg	1G1G	8-9	ND	2.0 ± 0.3	1.9 ± 0.4	2.4 ± 0.5	2.0 ± 0.4
	1G2G or 2G2G	12-15	ND	3.4 ± 0.9	2.9 ± 0.9*	3.8 ± 1.1	3.5 ± 1.0*
MMP-3t (10^2 U/mg)	5T5T or 5T6T	11	0.04±0.03	4.8 ± 1.5	3.4 ± 1.0*	5.5 ± 1.1	4.9 ± 1.2
	6T6T	8	0.01±0.00	3.9 ± 1.0	3.1 ± 0.8	4.5 ± 1.1	4.3 ± 1.3
TIMP-1 (ng/mg)	TT-♀ or T-♂	7	ND	25.7 ± 5.6	20.8 ± 4.0	22.8 ± 4.3	23.2 ± 3.6
	CT or CC-♀ or C-♂	12	ND	38.3 ± 5.7	35.0 ± 5.8	31.0 ± 5.3**	27.2 ± 3.7**
TIMP-2 (10^2 pg/mg)	GG	12	2.6 ± 0.2	6.4 ± 0.6	6.1 ± 0.5	4.2 ± 0.3***	4.6 ± 0.4***
	GA	7	2.3 ± 0.3	5.2 ± 0.5	5.5 ± 0.7	4.3 ± 0.6	4.5 ± 0.5
TNF-α (pg/mg)	GG	12-23	0.5 ± 0.2	5.5 ± 1.2	6.7 ± 1.7	73.3 ± 17.3**	43.8 ± 7.4***$^{\#}$
	GA or AA	6-7	0.8 ± 0.3	5.3 ± 2.1	10.9 ± 4.5	135.0 ± 61.8	58.7 ± 17.1*

SNP, single nucleotide polymorphism; IBD, inflammatory bowel disease; ND, not determined, below threshold of assays. Distribution of IBD and control tissue over genotypes is similar (Fisher's Exact test, not shown). IBD values in case of MMP-2 and MMP-9 are taken from inflamed and noninflamed tissue, otherwise only inflamed tissue was measured. MMP-9 expression by explants with CT or TT genotype is significantly lower when infliximab is added next to PWM, but is not reflected in depicted figures because PWM value is based on n = 8, PWM + infliximab on n = 5 and test is based on paired n = 5.***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ versus basal incubation,$^{\#\#\#}$ $P < 0.001$,$^{\#}P < 0.05$ versus PWM incubation, † $P < 0.05$ versus corresponding incubation of explants with alternative genotype. Statistical significance was not assessed between starting material and medium.

MMP-1, -3, and -9 production was enhanced or remained stable. However, mRNA levels of all four MMPs were similar or increased compared to basal incubation when PWM was added. Apparently, PWM activation may affect MMP posttranscriptional processes, particularly of MMP-2, resulting in the observed divergence of regulation of MMP protein production and secretion. When infliximab was added to basal medium, MMP-2 protein and mRNA levels remained similar, while MMP-1, -3, and -9 protein and/or mRNA were decreased. Clearly, the expression of MMP-2 in this explant culture system is not affected by TNF-α, whereas the basal expression of MMP-1, -3, and –9 protein appears to be substantially dependent on TNF-α, confirming previous observations on the regulation of these MMPs by this cytokine.[40] The downregulation of MMP-1, -3 protein by infliximab was paralleled by a decrease in corresponding mRNA in CD and control but not UC explants. This might point to aberrant regulation pathways of MMP-1 and -3 in UC compared to CD and controls, possibly related to specific pathogenic mechanisms. It can be argued that both absolute and relative levels of MMPs and TIMPs are physiologically relevant. Infliximab added to basal medium resulted in downregulation of the MMP-9 versus TIMP ratio, while MMP-2 versus TIMP remained constant. Importantly, addition of this drug also decreased the level of total MMP-1 and MMP-3 over TIMP, particularly TIMP-2, together with a downregulation of active MMP-1 and -3. These observations point to an antiproteolytic and matrix protective phenotype induced by infliximab. Conversely, addition of PWM increased the ratio of MMP-1, MMP-3, and MMP-9 to TIMP and also increased net MMP-1 and -3 activity, shifting the balance to a more proteolytic phenotype, possibly resulting in the observed significant epithelial cell targeted tissue degradation. Treatment with infliximab was able to partially prevent this PWM-induced increase, although not fully back to control levels. Therefore, our results indicate a clear relationship between expression of TNF-α and MMP-1, -3, and -9 net activity in CD, UC, and normal control tissue. This is corroborated by the finding of a positive correlation between TNF-α versus MMP-9/TIMP, MMP-3/TIMP-2, and TNF-α versus MMP-3 total activity in basal medium and of similar TNF-α and MMP/TIMP levels in corresponding incubations of IBD compared to control explants. However, despite less impressive differences in

TNF-α secretion, MMP activity levels were much higher in IBD compared to controls, stressing the importance of other proinflammatory pathways in upregulating MMPs. Our results on TNF-α reveal less enhancement compared to previous publications concerning TNF-α production by IBD mucosal biopsies.[5,41] Obviously, from surgical resection until culture our tissues had been exposed to more severe physical stress, perhaps upregulating TNF-α production processes. The duration of disease in our IBD study population was often several years, and as shown by Dionne *et al*,[42] this may downregulate TNF-α production. Alternatively, from many of our patients the removed bowel was affected by stricturing processes, perhaps paralleled by decreased capacity for production of TNF-α compared to solely inflamed tissue. Also, a large proportion of our patients were treated with corticosteroids and/or disease-modifying drugs like azathioprine or cyclosporine prior to surgical resection, possibly affecting TNF-α secretion during culture. Several studies have reported on functional SNP loci in the genes coding for MMP, TIMP, and TNF-α. We found increased production of corresponding protein or mRNA by explants with two C or at least one 2G, 5T or A allele at the MMP-2 -1306, MMP-1 -1607, MMP-3 -1613, and TNF-α –308 loci, respectively, confirming results in previous publications.[24,43-45] We also found an inhibitory effect of infliximab on MMP protein or mRNA production especially in explants with the CC and 1G2G or 2G2G genotype at SNP loci MMP-9 -1562 and MMP-1 -1607, respectively. These results may be relevant for future selection of infliximab responsive patients, but first have to be confirmed in larger studies. Experimental manipulation of cultured (human) intestinal IBD mucosa, as described here, may provide a new valuable tool in this respect. Despite the physical stress exerted on the tissue, the presence of bacteria, and the absence of fetal calf serum, we were able to preserve gross morphology up to 72 hours, with maintenance of crypts and cytokeratin-18-expressing epithelial cells, even when medium was not replenished. Possibly the presence of retinoic acid may play a central role, due to its anabolic effects on epithelial cells[46] and its attenuation of extracellular matrix degradation by stromal cells.[47] Given the large availability of surgical resection specimens, culture of IBD intestinal mucosa may offer an inexpensive way of monitoring drug efficacy, cytokine and proteinase expression, etc. In the past there have been several

reports describing potential mechanisms of infliximab action, i.e., downregulation of adhesion molecules on endothelial cells and production of IL-6 by fibroblasts,[48] antibody and/or complement-dependent (cellular) cytotoxicity,[49] induction of apoptosis in T lymphocytes,[50] and restoration of gut barrier function.[51] Our results demonstrate another potential mechanism of action, i.e., downregulation of excess MMP-1, -3, and -9 activity and parallel the observed downregulation of serum levels of various MMP members in rheumatoid arthritis[52] and CD patients[53] treated with infliximab. Our results reinforce the potential destructive capacities of MMPs in IBD pathogenesis, the pivotal role of TNF-α in regulating MMP-1, -3, and -9 activity, and the rationale for developing therapeutic intervention strategies utilizing specific MMP inhibitors for treating patients with CD or UC, as also previously shown in animal models.[18,54]

Acknowledgment

This study was supported by grant WS98-17 from the Netherlands Digestive Diseases Foundation.

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Chapter 4

Infliximab Treatment Influences the Serological Expression of Matrix Metalloproteinase (MMP)-2 and -9 in Crohn's Disease

Short title: Infliximab and gelatinases in CD

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This Study was published in Inflamm Bowel Dis 2007;13: 693-702

Abstract

Background/aims. Matrix metalloproteinases (MMPs) are actively involved in the pathogenesis of Crohn's disease (CD). We assessed the effect of the anti-tumor necrosis factor- α (TNF-α) monoclonal antibody infliximab on the *in vitro* and *in vivo* expression of MMP-2 and MMP-9 in CD.

Methods. Infliximab-treated fistulizing (n =10) or active disease (n = 7) CD patients, from an in-house study, and fistulizing CD patients (n = 42) and active CD patients (n = 24) from 2 placebo controlled studies were evaluated for serum MMP levels and clinical response. Biopsies were evaluated immunohistochemically for the MMPs. Whole blood cultures stimulated with lipopolysaccharide (LPS)/infliximab were evaluated for MMP mRNA and protein levels.

Results. Serum MMP-2 levels in CD patients increased during follow-up, similarly in responders and nonresponders, by infliximab. Immunohistochemistry showed no clear MMP-2 change in biopsies. Serum MMP-9 levels, however, showed a consistent pattern of decrease in most CD patients, particularly in those responding, and MMP-9-positive polymorphonuclear leukocytes in biopsies also decreased by infliximab. LPS stimulation of whole blood increased the MMP-9 levels in plasma significantly in CD patients and controls, but infliximab had no effect on the secretion. Long-term LPS stimulation raised leukocyte MMP-9 mRNA levels 16-fold and infliximab inhibited this induction by 80%.

Conclusions. Infliximab treatment increases MMP-2 and decreases MMP-9 in serum of patients with CD, the latter also in the intestine, which extends and confirms our previous *ex vivo* explants observations. However, these changes were not strictly associated with the response to treatment. The enhanced leukocyte MMP-9 expression in CD seems to be regulated by TNF-α.

Introduction

Infliximab, a chimeric IgG1 anti-tumor necrosis factor (TNF)-α monoclonal antibody, is a successful immunotherapeutic agent for Crohn's disease (CD). The treatment with infliximab results in a high clinical efficacy, rapid onset of action, and prolonged effect in patients with moderate to severe active CD, which have not responded to conventional therapy, and in fistulizing CD patients. Simultaneously, the quality of life of these patients is essentially improved.[1-3] The proposed immunological mechanisms of infliximab include the suppression of TNF-α bioactivity and the lysis of TNF-α-producing cells, such as monocytes and lymphocytes, via complement fixation, antibody-dependent cellular cytotoxicity (ADCC), and Fc portion binding of the IgG1 antibody. Furthermore, infliximab downregulates mucosal Th1 cytokines, reduces the expression of IFN-γ and other inflammatory molecules, such as intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1.[4-6]

Matrix metalloproteinases (MMPs) compose a family with over 20 members of Zn^{2+}-containing neutral proteinases.[7] Usually, MMPs are synthesized as preproenzymes and are secreted in a proenzyme form that requires proteolytic cleavage for activation in most cases.[8,9] The activity of MMPs is precisely regulated within tissues by the balance between zymogen activation and enzyme inhibition. Factors that regulate activity of the MMPs include endogenous inhibitors, α-macroglobulins, and tissue inhibitors of metalloproteinases (TIMPs) and the MMPs themselves.[10,11] MMPs are implicated in the inflammatory response, wound healing, tissue remodeling, cell growth, migration, apoptosis, cell-cell communication, tumor invasion, and metastasis.[8,12-15] MMPs exert their activity by the degradation of a class of biological molecules that include not only the components of extracellular matrix (ECM) but also an increasing family of bioactive modulators, such as cytokines, growth factor receptors, other proteinases, coagulation factors, chemotactic molecules, and adhesion molecules.[14] In the pathogenesis of CD, MMPs are believed to be associated with the injury of gut tissue mediated by TNF-α and Th1 cytokines. One of the mechanisms by which TNF-α causes intestinal tissue injury is believed to be the enhancement of the MMP production at local sites.[16-18] MMP-2 (72 kDa, gelatinase A) and MMP-9 (92

kDa, gelatinase B) are the two members of the gelatinase subgroup of MMPs. The substrates of MMP-2 and MMP-9 specifically include not only basement membrane (BM) type IV collagen and other components like gelatin, collagen type I, V, VII, X, elastin, laminin, and fibronectin, but also numerous bioactive molecules, such as fibroblast growth factor receptor (FGFR)-1, prointerleukin (IL)-1, and ICAM-1.[19-22] Previous studies showed that MMP-2 and -9 are actively involved in the pathophysiological processes, including fistula formation, in the intestine of inflammatory bowel disease (IBD) patients.[23-30] After treatment with infliximab the elevated levels of MMP-1 and -3 in serum of patients with rheumatoid arthritis were reported to be reduced.[31] The role of MMPs in the treatment of CD patients with infliximab, however, is still poorly understood. Recently, we showed that infliximab induces a matrix protective MMP-phenotype in *ex vivo* cultures of mucosa from patients with inflammatory bowel disease.[32] In the present study we explored further this relationship between the clinical efficacy of infliximab and the expression levels of MMP-2 and -9 in patients with CD and describe the *in vivo* and *in vitro* regulation of the expression of these 2 gelatinases by infliximab.

Patients and methods

Clinical studies

In the present study we included patients that participated in an expanded access program for infliximab treatment of CD in our institute, i.e., in-house study,[3] and a subgroup of patients that participated in an international multicenter, placebo-controlled trial of infliximab either for the treatment of fistulas in patients with CD or for the treatment of active CD.[1,2] The in-house study was approved by the medical ethics committee and patients were only included after informed consent. The eligibility of patients in these studies was described previously.[3,33] Briefly, the age of confirmed patients with CD had to be between 18 and 65 years. For inclusion in the fistula treatment groups, patients had to have single or multiple draining abdominal or perianal fistulas of at least 3 months' duration. Patients who had had CD for at least 6 months, with CD Activity Index (CDAI) scores equal or above 220, were eligible for the treatment with infliximab for active CD. Analyses of efficacy evaluated the number of patients with a reduction of half or more in the number of

draining fistulas from baseline as responders or those with complete healing (defined as the absence of any draining fistulas) as healers at 2 consecutive visits. Changes in scores of the CDAI and the open fistulas scores were also evaluated. Failure of treatment was defined as changes in medication that were not permitted in the protocol, surgery related to CD, or no return for follow-up visit.

Protocol 1. Fistulas

Within 2 weeks of screening, eligible patients (n = 10, one patient also with active disease) for the in-house study received infliximab 5 mg/kg (body weight) and patients from the international trial were randomly assigned to receive 1 of 3 treatments at weeks 0, 2, and 6: placebo (n = 14) or 5 or 10 mg/kg of infliximab (total n = 28). Study drug was administered intravenously over a 2-hour period. After the first infusion of study medication, patients returned for clinical and laboratory assessments at weeks 2, 6, 10, 14, and/or 18. Serum samples were collected, if possible, at each study visit through week 18.

Protocol 2. Active disease

Patients in the in-house study received a single dose of 5 mg of infliximab per kilogram of body weight (total n = 7) in an intravenous infusion, administered over a 2-hour period. Disease activity according to the CDAI and/or blood serum samples were assessed at days 0, 3, weeks 2, 4, 8, and 12. In the international study patients were randomly assigned to receive a single dose of either placebo (n = 7) or 5, 10, or 20 mg/kg of infliximab (total n = 17). Disease activity was assessed and serum samples were collected at weeks 0 and 4.

***In vitro* study**

Patients, volunteers, and blood samples

Patients with CD (n = 7) were treated with infliximab for fistulizing and/or active disease in the in-house study. Blood from healthy volunteers (n = 5) was obtained, with their permission, from the central blood transfusion laboratory. Heparinized blood samples were obtained from patients before and 2 hours after a single infusion of infliximab of 5 mg/kg over a 2-hour period. For the blood samples from

the healthy volunteers a concentration of 75 µg infliximab per mL blood was added. At 37 °C and 5% CO_2, whole blood samples with/without infliximab were stimulated with/without lipopolysaccharide (LPS) (Sigma, St. Louis, MO) at 0.1 µg/ml blood for 1.5–24 hours for MMPs and TNF-α mRNA and protein determinations.[34] The plasma was subsequently separated from the blood sample by centrifugation at 4 °C, and stored at -70 °C until further analysis. Leukocytes isolation was performed by adding lysis buffer containing 0.16 M NH_4Cl, 10 mM $KHCO_3$, and 0.01 mM K_2-EDTA (pH 7.4 at 0°C) to the samples. After erythrocytes were degraded the sample was centrifuged at 4 °C and lysis was repeated to obtain pure leukocytes. The leukocytes were immediately used to isolate RNA.

Determination of MMPs by ELISA

MMP-2 and MMP-9 levels in the samples were measured by our highly specific enzyme-linked immunosorbent assays (ELISA), which measure the total of proenzyme, active-, and inhibitor-complexed forms of the respective MMP, as described previously.[30,32,35] In brief, a polyclonal anti-MMP-2 antibody or monoclonal anti-MMP-9 antibody was used as catching antibody and appropriately diluted samples were incubated overnight at 4 °C. Immunodetection of MMP-2 was performed using polyclonal anti-MMP-2 followed by biotin-labeled goat antirabbit-IgG and of MMP-9 with biotin-labeled polyclonal anti-MMP-9 antibody. After incubation with avidin-peroxidase the chromogenic substrate 3,3,5,5-tetramethyl benzidine in the presence of hydrogenperoxide 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 mL serum or plasma.

Immunohistochemical staining for MMPs

Standardized colonic biopsies of 6 patients from the in-house study (4 with fistulizing and 2 with active disease) were obtained at the start of the study, as well as at day 3 and week 2 of follow-up. 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.[30] In brief, paraffin tissue sections,

treated with proteinase K for MMP-2 antigen retrieval, were incubated with rabbit polyclonal antihuman MMP antibodies, similar to those used in the ELISAs. Subsequently, the sections were incubated with biotinylated goat antirabbit Ig, peroxidase-labeled streptavidin, and stained with 3-amino-9-ethylcarbazole and hematoxylin. Control sections incubated with preimmune 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, 4 = all cells or areas of tissue strongly positive.

Reverse transcription–polymerase chain reaction (RT-PCR)

Oligonucleotide primers (Table 1) for RT-PCR were derived from the DNA sequence database of the NCBI (Bethesda, MD). Primer sets were designed using the Primers3 Output computer program (Whitehead Institute for Biomedical Research, Cambridge, MA). The MMP-2 and MMP-9 PCR products span 3 introns to prevent interference of contamination by genomic DNA. Specificity of the primers was checked with the NCBI BLAST program. β2-microglobulin (β2-M) was used as a control to normalize PCR signals from the different samples. Total RNA was isolated from blood samples by the method of Chomczynski and Sacchi.[36] The integrity and quality of the purified RNA were analyzed by 1.5% agarose gel/ethidium-bromide staining and the 260/280 nm absorbance ratio. Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase was used for cDNA synthesis from DNAse-treated RNA. The PCR was started at 94 °C for 3 minutes, followed by 30 cycles for MMP-2 and MMP-9, and 28 cycles for β2-M. Each cycle consisted of a denaturation step (at 94 °C for 30 sec), an annealing step for 45 sec (at 56 °C for MMP-2 and β2-M, 59 °C for MMP-9), and extension step (at 72 °C for 1 min), followed by a final elongation step (at 72 °C for 7 min), as described previously.[32] The reaction was performed in a Whatman T Gradient cycler (Biometra, Goettingen, Germany) and the amplified products were electrophorized on 1.5% agarose gels containing ethidium-bromide (0.5 g/mL) and visualized under

Table 1. Oligonucleotide primers for RT-PCR.

MRNA	Gene	Sense and Antisense Primer	Product Size
NM-004530	MMP-2	AGGATCATTGGCTACACACC AGCTGTCATAGGATGTGCCC	535
NM-004994	MMP-9	CGCAGACATCGTCATCCAGT GGATTGGCCTTGGAAGATGA	406
NM-000594	TNF-α	CCCCAGGGACCTCTCTCTAA GGAAGACCCCTCCCAGATAG	413
NM-004048	β2-M	CCAGCAGAGAATGGAAAGTC GATGCTGCTTACATGTCTCG	269

RT-PCR, reverse transcription–polymerase chain reaction; MMP, matrix metalloproteinase.

ultraviolet light. RT-PCR, which contained RNA but not M-MLV reverse transcriptase, was used to check contamination with genomic DNA. The Scion imaging program (Frederick, MD, www.scioncorp.com) was used to semiquantify the band density in the gels, expressed in arbitrary units (AU).

Statistical analysis

The results of the MMP ELISAs are given as mean ± SEM, the clinical, immunohistochemical, and mRNA data are presented as median with interquartile range. The Wilcoxon signed-rank test or the paired Student *t*-test was used to evaluate difference between paired data and the Mann–Whitney U-test or the Student *t*-test for unpaired data, where applicable. Differences were considered significant when $P \leq 0.05$.

Results

Clinical studies

MMP-2

The serum MMP-2 level in patients with fistulas from the in-house study ($n = 6$) responding to treatment with infliximab showed a steady increase from 605 ± 78 (ng/mL) at day 0 up to 834 ± 46 at week 14 ($P = 0.08$). Correspondingly, the open/draining fistulas score in these patients was decreased at the end of follow-

up from 3 (2–6) to 1 (0–1) (P = 0.03); most of these responders already had signs of improvement at week 2. Except for the first 3 days, where the level of MMP-2 in the nonresponders of the patients with fistulas (n = 4) decreased due to 1 outlier, also the nonresponding patients showed a gradual increase up to 649 ± 107 at the end of follow-up. The open fistulas scores in this group remained at 1, and all these nonresponding patients had genitourinary fistulas. Similarly, from day 0 to week 10 in the international study the serum MMP-2 level in fistulizing CD patients increased in both placebo (n.s.) and infliximab (P = 0.003) treated patients, and in both responders/healers and nonresponders/nonhealers (0.02 < P < 0.08), with a general decrease after week 10. Baseline MMP-2 levels at inclusion were comparable between all subgroups (Fig. 1). Also in CD patients with active disease (n = 7) from the in-house study who responded to the treatment with infliximab, the improvement of disease activity was accompanied by a significant increase of the serum MMP-2 level from 364 ± 54 (ng/mL) at day 0 to 656 ± 53 at week 8 (P = 0.03). The CDAI decreased significantly from 365 (264–461) down to 50 (10 –189)

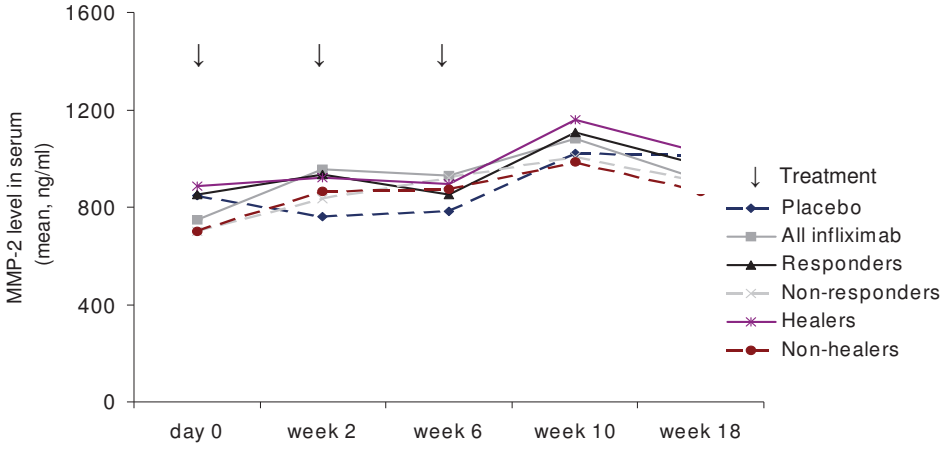

Figure 1. Serum MMP-2 levels in all subgroups of patients with fistulas from the international study showed a similar pattern, i.e., slightly increasing during the follow-up, with a slight decrease at the end. MMP: matrix metalloproteinase. Placebo: n = 14; all infliximab: n = 28; responders: n = 22; nonresponders: n = 19; healers: n = 18; nonhealers: n = 23.

at the corresponding timepoints (P = 0.04). At the end of follow-up, i.e., week 12, the MMP-2 level started to decrease and the CDAI started to increase again. One patient showed no improvement with the infliximab treatment and the serum MMP-2 level in this patient remained stable over time. The serum MMP-2 level in patients with active disease from the international study did not show consistent and significant changes at the end of 4 weeks follow-up compared with day 0 in both placebo and infliximab-treated groups (Table 2), although at higher dosages a tendency to increase was clearly discernable.

Table 2. Serum MMP-2 and -9 levels in patients with active disease included in the international study.

		Placebo *n* = 7	Infliximab 5 mg/kg *n* =4	Infliximab 10 mg/kg *n* = 6	Infliximab 20 mg/kg *n* = 7
MMP-2	Day 0	780 ± 170	935 ± 411	658 ± 126	780 ± 80
	Week 4	715 ± 225	879 ± 160	998 ± 118	898 ± 172
MMP-9	Day 0	344 ± 79	180 ± 62	286 ± 111	255 ± 48
	Week 4	207 ± 46	194 ± 65	232 ± 86	135 ± 29

MMP, matrix metalloproteinase. MMPs presented in ng/mL (mean ± SEM).

MMP-9

The MMP-9 serum level of the in-house patients with fistulizing disease in both responders and nonresponders was hardly affected by the infliximab therapy, although at the end of follow-up a decrease was noticed from 297 ± 41 to 267 ± 76 and from 441 ± 48 to 240 ± 61 ng/mL, respectively. In the international study there seemed to be a general trend to a decreased MMP-9 level where the infliximab-treated patients and responders/healers seemed to have a slightly lower level at week 6 to 18 than the placebo treated and nonresponding/nonhealing patients, although no statistical significance was reached (Fig. 2). Active disease patients from the in-house study who responded to infliximab had a decreased MMP-9

serum level from day 3 after treatment onwards. The MMP-9 serum level fell from 419 ± 88 at the beginning of the study down to 236 ± 26 at week 4 ($P = 0.05$), and remained at this lower level to the end of the follow-up, 230 ± 52 ($P = 0.05$), accompanying the decrease in CDAI. The MMP-9 level in the 1 patient who did not respond to the treatment also showed a reduction of MMP-9 serum levels during the follow-up. In the international study the levels of MMP-9 in the active CD patients were also decreased in both the placebo and infliximab-treated groups at the end of the follow-up, except for the 5 mg/kg infliximab group, but no statistical significance was reached (Table 2).

Immunohistochemical results

A patchy and relatively strong positive immunoreaction to MMP-2 was present in the ECM of submucosa in noninflamed tissues (Fig. 3B). In inflamed tissues a positive staining of MMP-2 was observed in endothelial cells and the ECM of the lamina propria (Fig. 3B,C). There were no major differences between patients with fistulas or with active disease. Overall, the immunohistochemical expression pattern of MMP-2 did not seem to change by the treatment with infliximab therapy (data not shown). The immunoreactivity for MMP-9 was predominantly present in the polymorphonuclear leukocytes (PMNL). A relatively high PMNL positive staining for MMP-9 was observed in the tissues before treatment [median score 2 (IQR 1–2.5)]. Follow-up biopsies after treatment with infliximab revealed a decreased intensity of MMP-9 staining already at day 3 [1 (1–1.8), n.s.], which was even lower at week 2 [0.5 (0–1.3), $P < 0.05$] in 5 out of 6 patients, 4 of them with a good clinical response (Fig. 4B,C). Interestingly, in most of the tissue sections we found enteroendocrine cells to be positive for MMP-9, independent of treatment with infliximab (Fig. 4D).

***In vitro* study**

Whole blood cultures revealed that the levels of MMP-2 in plasma of both CD patients and controls were not affected by stimulation with LPS or with LPS in the presence of infliximab for 1.5 or 24 hours. However, the levels of MMP-2 in the plasma of CD patients were in general lower than that in healthy controls (Table 3).

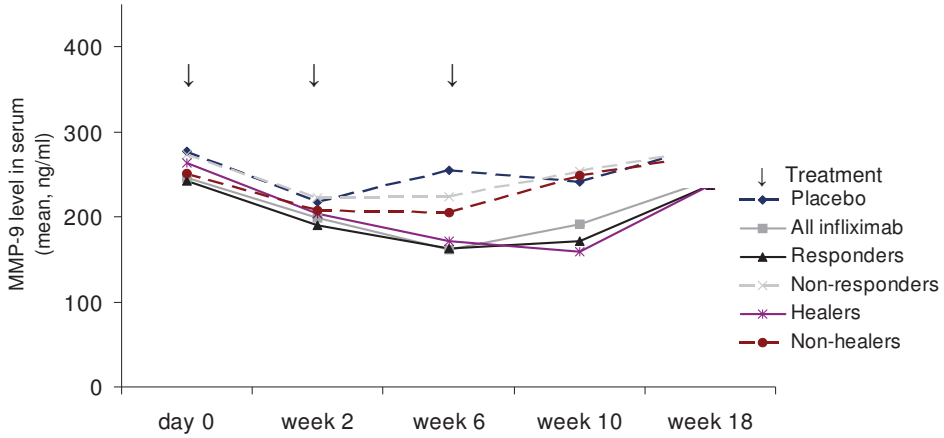

Figure 2. In contrast to the serum levels of MMP-2, MMP-9 levels in patients with fistulas from the international study showed a trend to decrease in all groups during the follow-up, with a slight increase at the end. MMP: matrix metalloproteinase. Placebo: *n* = 14; all infliximab: *n* = 28; responders: *n* = 22; nonresponders: *n* = 19; healers: *n* = 18; nonhealers: *n* = 23.

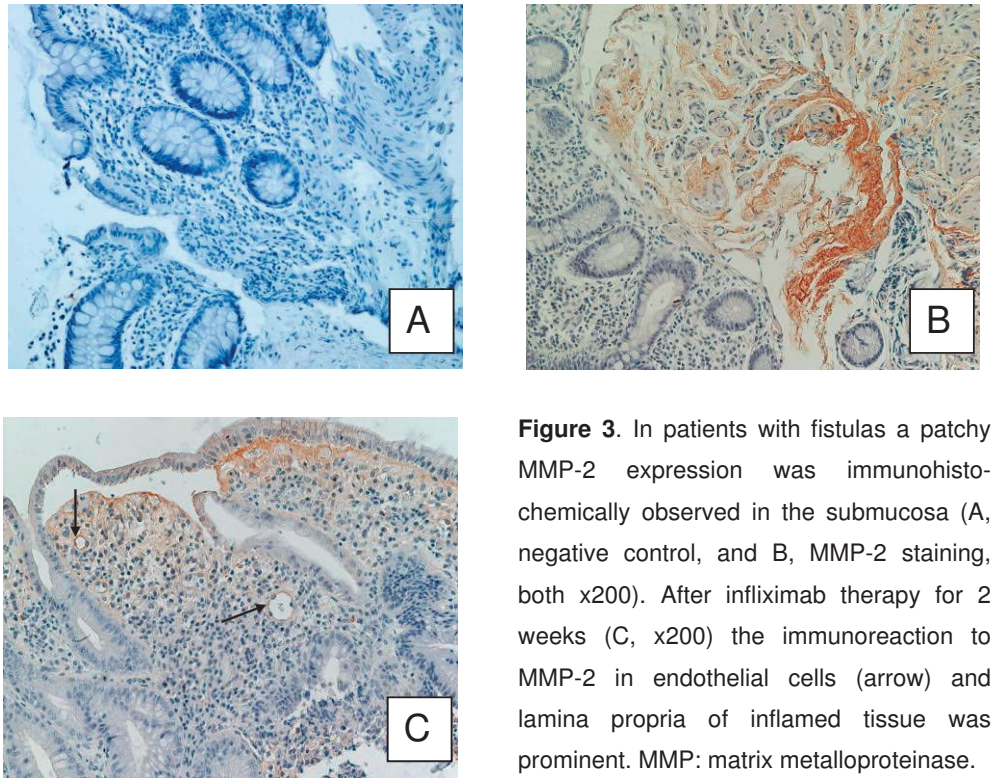

Figure 3. In patients with fistulas a patchy MMP-2 expression was immunohistochemically observed in the submucosa (A, negative control, and B, MMP-2 staining, both x200). After infliximab therapy for 2 weeks (C, x200) the immunoreaction to MMP-2 in endothelial cells (arrow) and lamina propria of inflamed tissue was prominent. MMP: matrix metalloproteinase.

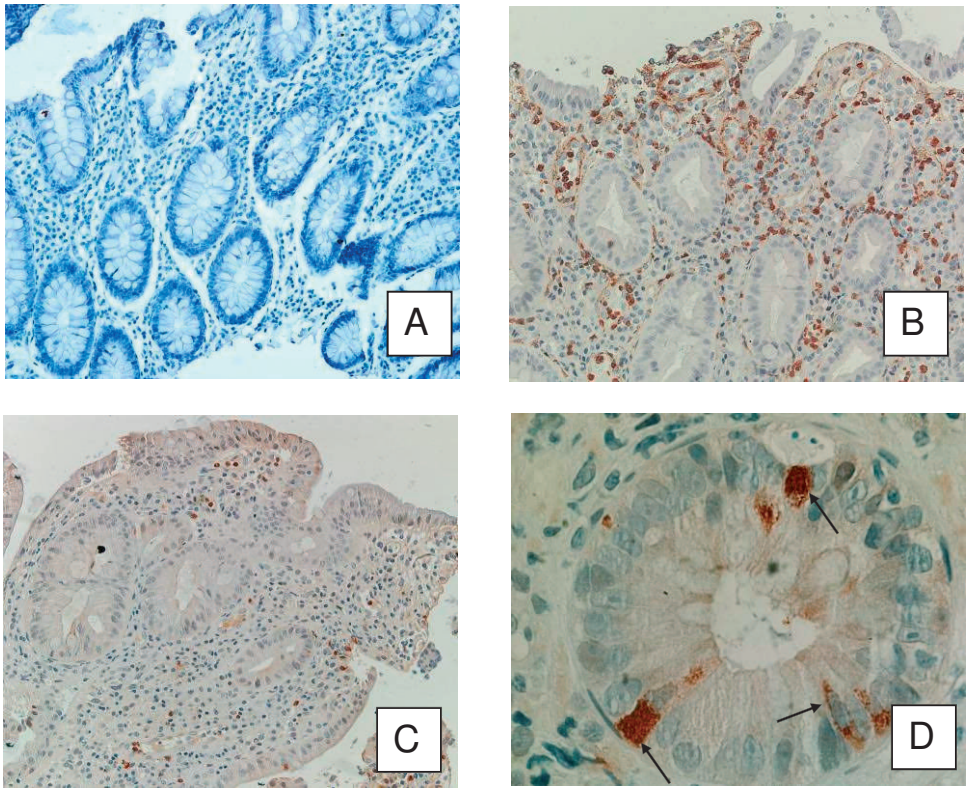

Figure 4. MMP-9 immunostaining was predominantly present in polymorphonuclear leukocytes within the lamina propria (A, negative control, and B, MMP-9 staining, both x200). After treatment of this patient with infliximab for 2 weeks MMP-9 staining was found to be decreased (C, x200). The positive immunoreaction of MMP-9 in enteroendocrine cells (arrow) was frequently observed (D, x1000). MMP: matrix metalloproteinase

With RT-PCR no detectable MMP-2 mRNA level was found in leukocytes of both CD patients and healthy volunteers. In contrast to the levels of MMP-2, MMP-9 levels in patients' plasma were higher, compared with those in healthy volunteers. After a 1.5-hour LPS stimulation the levels of MMP-9 were significantly increased in CD patients and in controls, both more than 2-fold higher than the unstimulated cultures, with the increase in the CD patients significantly higher than that in the healthy volunteers (P = 0.05). Infliximab was found not to affect MMP-9 protein levels of this short-term LPS stimulation (Table 3). After 24 hours, LPS stimulation

Table 3. Plasma MMP levels from the *in vitro* whole blood cultures of CD patients and healthy volunteers

	Patients (n = 7)		Volunteers (n = 5)	
MMP-2	1.5h	24h	1.5h	24h
Blank	380 ± 108	477 ± 148	936 ±200	771 ± 117
LPS	397 ± 140	639 ± 144	887 ± 120	833 ± 203
Infliximab+LPS	462 ± 52	421 ± 98	913 ± 346	718 ± 119
MMP-9	1.5h	24h	1.5h	24h
Blank	541 ± 209	315 ± 55	126 ± 18	194 ± 31
LPS	1132 ± 242**	848 ± 204*	364 ± 75*	679 ± 106*/***
Infliximab+LPS	1296 ± 412*	878 ± 327	268 ± 46*	550 ± 63*/***

MMP, Matrix Metalloproteinase; CD, Crohn's disease; LPS, lipopolysaccharide. *P <0.05, **P < 0.01 versus blank; ***P < 0.05 versus 1.5h- incubation. MMPs presented in ng/mL (mean ± SEM).

did not further promote leukocytes to synthesize/secrete MMP-9 in CD patients, but in the healthy volunteers LPS stimulated the MMP-9 protein synthesis/secretion about 2-fold, which was significantly higher in comparison with the level after 1.5 hours (P < 0.05). Infliximab did not affect the MMP-9 protein secretion after 24 hours in either patients or healthy volunteers. Interestingly, the 1.5-hour LPS stimulation hardly changed the respective MMP-9 mRNA level compared to that of the blank samples, whereas TNF-α mRNA was increased 13-fold (Table 4). More MMP-9 mRNA was transcribed in leukocytes after 24 hours stimulation with LPS, raising up to 16-fold. This transcription of MMP-9 mRNA was mediated by TNF-α

Table 4. MMP-9 and TNF-α mRNA levels in cultured leucocytes from healthy volunteers

mRNA	1.5h LPS	24h LPS	24h LPS + Infliximab
MMP-9	0.5 (0.1–1.4)	16 (2–47)	3 (3–7)
TNF-α	13 (10–121)	4 (3–9)	2 (0.5–4)

MMP, matrix metalloproteinase; LPS, lipopolysaccharide. The results represent 4 experiments, with mRNA levels expressed in median relative densitometry units (inter quartile range) in comparison with blank samples.

as infliximab downregulated the mRNA level by almost 80%. At the same time, the TNF-α mRNA level in the 24-hour samples was enhanced by only 4-fold and even lower, i.e., 2-fold, in the presence of infliximab.

Discussion

Treatment with infliximab is very effective in patients with active or fistulizing CD, although the mechanism(s) of action have not yet been fully elucidated.[1,2,6] MMPs have been implicated in both disease phenotypes and infliximab was recently found to be inducing a mucosa protective MMP-phenotype in *ex vivo* mucosal IBD explants.[23-30,32] In the present study, we found that these infliximab-induced alterations in MMPs are to a large extent also reflected in the serum and in intestinal biopsies. We found an increase of serum MMP-2 in both fistulizing and active CD patients by the treatment with infliximab, in comparison to baseline, with a decline at the end of follow-up after cessation of treatment. The cause of the increase in MMP-2 might be related to the turnover of the intestinal tissue in CD, especially the remodeling of the ECM components. CD is a chronic and recurrent inflammation of the alimentary tract, where remission and relapse of the disease alternate and almost inevitably occur. In the inflammatory process the destruction and healing of tissue seems to occur simultaneously. This could partially explain why the increase of serum MMP-2 is not strictly related to the criteria of clinical improvement. During these processes there is formation of granulation tissue, especially at ulcerative and fistulizing sites, where the remodeling of tissue actively takes place. This granulation tissue differs from the normal tissue in composition of cells and matrix components, containing many fibroblasts and endothelial cells. In addition, we previously showed that in the inflamed area MMP-2 is significantly increased.[30] The proliferation, differentiation, and (neo)angiogenesis are highly promoted by stimulation through induced growth factors, such as transforming growth factor (TGF)-β, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF). The increase of MMP-2 at local sites is probably to meet the demand of active ECM turnover. Ågren[37] also believed that MMP-2 is important during the prolonged remodeling phase in wound healing. Apparently, at

moments of high tissue demand for MMP-2 the serum levels are low and at inclusion of controlled treatment protocols these serum levels start to increase, not strictly related to response. The sequestration of MMP-2 to ECM of intestinal IBD tissue may also partially contribute to the paradoxical phenomenon, i.e., the elevated expression of MMP-2 in inflamed tissue and low level in the circulation.[18] MMP-2 is constitutively expressed by many cells, especially mesenchymal cells, such as fibroblasts and endothelial cells, and has a ubiquitous tissue distribution.[20] TNF-α and other proinflammatory cytokines seem not to be major promoting factors for the expression of MMP-2 because the gene promoter lacks TPA-responsive element (TRE). TGF-β, however, is regarded as a stimulator for the expression of MMP-2, which might be relevant to the remodeling processes.[20] Our *in vitro* results do confirm this since the levels of MMP-2 in plasma were not affected by the incubation of leukocytes with immunomodulators like LPS and/or infliximab. Serum MMP-9 levels, in contrast to MMP-2, in the CD patients were found to be reduced by the treatment, with an increase again at the end of the follow-up. Similar to the duration of the increase of the MMP-2 level, the decrease of MMP-9 also lasted for a time period that coincides with the duration that infliximab is maintained at a detectable level in the circulation.[6] MMP-9 is thought to be an active participator in the process of inflammation in CD, especially in the acute phase. Unlike other MMPs, MMP-9 is normally stored in secondary and tertiary granules of neutrophils poised for rapid release to participate in the reaction of the host to exogenous and endogenous stimulation. MMP-9 not only lyses components of the ECM, but also influences the generation or activation of c-x-c and other chemokines, which attract neutrophils to migrate across the BM of capillaries to inflammatory sites.[38] Kirkegaard *et al*[29] recently found MMP-9 to be markedly upregulated in and contribute to intestinal fistula formation in CD. During the evolvement of tissue repair, overexpression of MMP-9 has been speculated to prevent the healing process.[8] In contrast, Salo *et al*[39] concluded that MMP-9 plays a prominent role because it participated in every step of the healing process, including detachment of epithelial cells from the basal membrane, rolling of cells to the wound matrix, and remodeling of the granulation tissue. The neutrophil is the most important source of MMP-9 in the acute phase of inflammation.[40] In the

present study, immunohistochemical evaluation showed that MMP-9 predominately existed in the neutrophils and to a lesser extent in the ECM of severely inflammatory regions. The reduction of the MMP-9 expression in intestinal tissue from the infliximab-treated CD patients is probably related to the decrease in the number of the inflammatory cells, especially neutrophils and monocytes/ macrophages.[41,42] In addition, our previous *ex vivo* mucosal IBD explants study revealed that within 72 hours infliximab is able to markedly reduce the MMP-9 synthesis/secretion also.[32] MMP-9 was also found to be present in enteroendocrine cells. The significance of this observation needs to be elucidated further. Perhaps there is a similarity with the presence of MMP-7 in intestinal Paneth cells, which is believed to be related to the activation of α-defensin.[43,44] Proinflammatory cytokines, such as TNF-α, IL-1β, are able to activate the MMP-9 gene through nuclear factor(NF)-κβ to enhance MMP-9 production.[39,45] LPS stimulates monocytes to express MMP-9, which is partly dependent on TNF-α because neutralization of TNF-α significantly downregulated the production of MMP-9.[46] Our *in vitro* study also showed that MMP-9 is released from leukocytes of both CD patients and healthy volunteers after short-term 1.5 hours LPS incubation. The release of MMP-9 from leukocytes of the CD patients was significantly higher than from healthy volunteers. Most likely, the abundance of MMP-9 in the CD neutrophils occurred during the process of their maturation activation in response to different stimulators, such as TNF-α and bacterial products.[47,48] TNF-α seemed not to be involved in the process of MMP-9 secretion by neutrophils *in vitro*, as infliximab did not affect the level of MMP-9 in plasma. The transcription of MMP-9 mRNA was found to be hardly affected by short-term LPS stimulation, probably because of the increased production of other immediate response mRNAs like that of TNF-α. Further incubation with LPS for 24 hours increased the level of MMP-9 significantly in healthy volunteers rather than in CD patients. The MMP-9 mRNA was also strongly upregulated. Infliximab did suppress this increase of MMP-9 mRNA, therefore we conclude that TNF-α is involved in the promotion of the transcription of MMP-9 mRNA. In the CD patients the MMP-9-producing blood cells probably lost their potential ability to further induce MMP-9 synthesis. We speculate that monocytes are the main MMP-9-producing blood cell type responding to the

long-term LPS incubation in this study. Pugin *et al*[46] previously reported that the maximal level of monocytic MMP-9 synthesis was between 24-48 hours after LPS stimulation. Other studies also showed that LPS could activate monocytes to express the MMP-9 gene and protein, although they did not implicate TNF-α in their studies.[49,50] The MMP-9 changes we observed in the serological follow-up of our patients are, however, most likely a reflection of what is happening at the mucosal level, also based on our explant studies, and less so an immediate effect of infliximab on circulating blood cell MMP-9 production and secretion.

In conclusion, the serum MMP-2 and MMP-9 level in CD patients display an inverse changing pattern, i.e., an increase of MMP-2 and a decrease of MMP-9 during the treatment with infliximab, although not strictly related to the clinical effect of infliximab. The enhanced leukocyte MMP-9 expression in CD seems to be regulated by and responsive to TNF-α mediation. These clinical findings reinforce previous observations in experimental models showing MMP-2 to be protective and MMP-9 to be enhancing dextran sodium sulfate-induced colitis, rendering the latter a potential therapeutic target.[51-53]

Acknowledgments

We thank Drs. Roeland Hanemaaijer and Jan Verheijen from TNO Quality of Life, Biomedical Research, Leiden, The Netherlands for technical assistance, Prof. S.J. van Deventer from the Department of Gastroenterology and Hepatology, Academic Medical Center, Universiteit van Amsterdam, Amsterdam, The Netherlands, and Profs. K. Geboes and P. Rutgeerts from the Departments of Pathology and Gastro-enterology-Hepatology, Universitaire Ziekenhuizen, Leuven, Belgium, for permission to include patients from their centers. We thank S. Marks from Medical Affairs Europe, Centocor, Leiden, The Netherlands, for support to perform the study.

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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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This Study was published in Dig Liver Dis 2007; 39:733-739

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

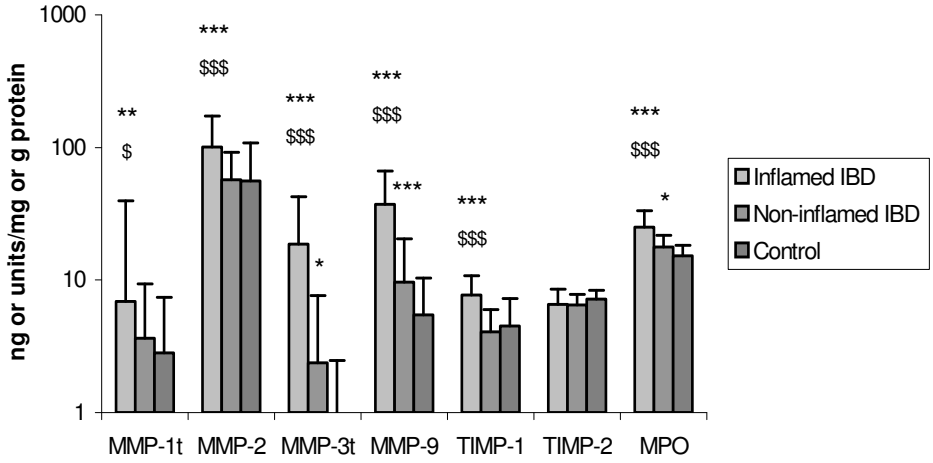

Figure 1. Total MMP, TIMP and MPO protein/activity expression (median + 75th percentile) by inflamed and non-inflamed IBD vs. normal control intestinal mucosa. *n* = 28, 25, 16 (MMP-1t); 70, 50, 22 (MMP-3t); 178–179, 140, 62 (MPO, MMP-2, -9 and TIMP-1, -2) for inflamed IBD, non-inflamed IBD and controls, 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).

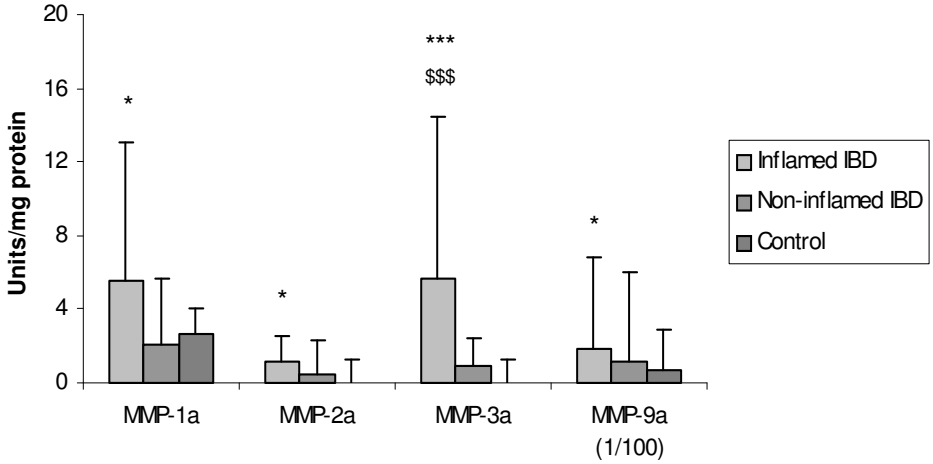

Figure 2. Net MMP activity (median + 75th percentile) in inflamed and non-inflamed IBD vs. macroscopically normal control intestinal mucosa. n = 28, 25, 16 (MMP-1a); 70, 52, 22 (MMP-2, -3, -9a) for inflamed IBD, non-inflamed IBD and controls, respectively. $^{***}P < 0.001$, $^{*}P < 0.05$ vs. controls; $^{\$\$\$}P < 0.001$ 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).

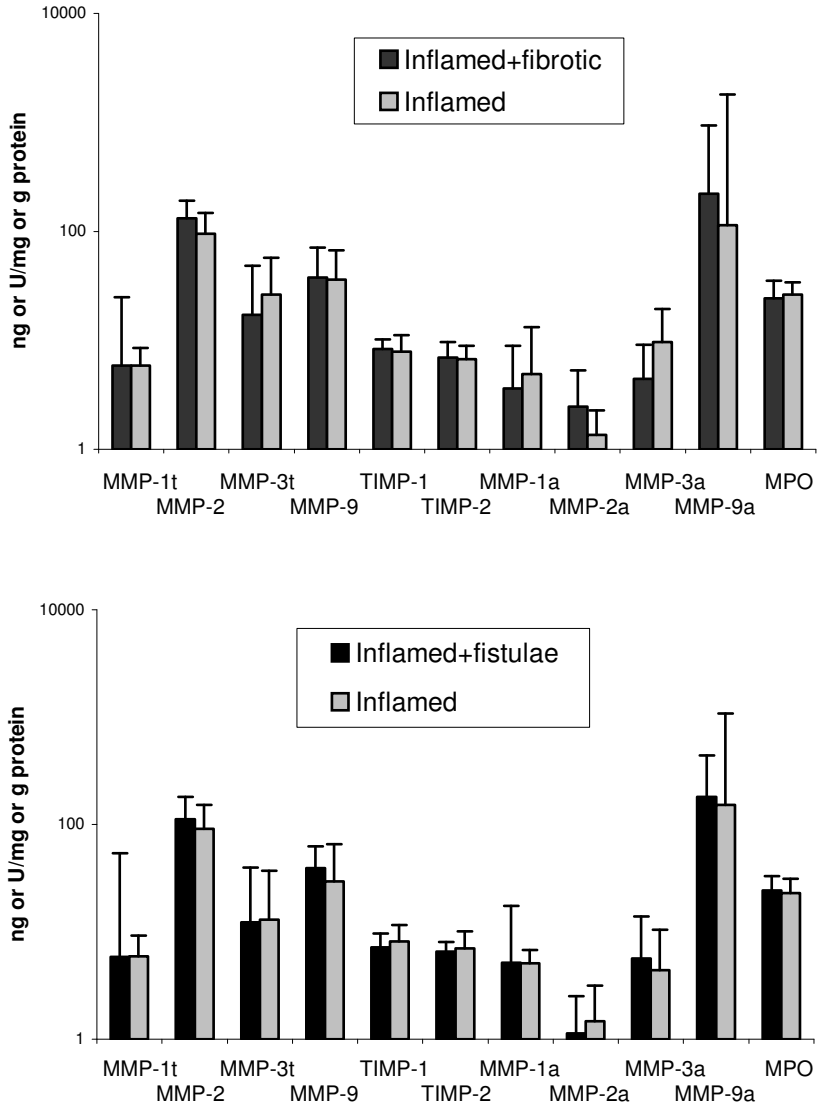

Figure 3. MMP, TIMP and MPO protein/activity expression (median + 75th percentile) by inflamed CD intestinal mucosa with or without extensive fibrosis (a) and with or without fistulizing disease during follow-up (b) (unpaired data). *n* = 7–10 (MMP-1t, MMP-1a); 16–31 (MMP-2a, MMP-3t, MMP-3a, MMP-9a);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

Acknowledgments

Drs. R. Hanemaaijer and J.H. Verheijen from TNO Quality of Life, Biomedical Research, Leiden, The Netherlands. This study was supported by the Netherlands Digestive Diseases Foundation Grant WS98-17.

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Chapter 6

Role of Matrix Metalloproteinase, Tissue Inhibitor of Metalloproteinase and Tumor Necrosis Factor-α Single Nucleotide Gene Polymorphisms in Inflammatory Bowel Disease

Short title: Role of SNPs in IBD

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This Study was published in World J Gastroenterol 2007;13:2960-2966

Abstract

Background/aims. To study the (functional) relevance of single nucleotide polymorphisms (SNPs) in genes encoding matrix metalloproteinases (MMP)-1, -2, -3, -9, tissue inhibitors of metalloproteinases (TIMP)-1, -2 and tumor necrosis factor (TNF)-α in the etiopathogenesis of inflammatory bowel diseases (IBD), that may enhance susceptibility and/or disease severity.

Methods. Genomic DNA from 134 Crohn's disease (CD), 111 ulcerative colitis (UC) patients and 248 control subjects was isolated from resected intestinal tissue or blood. Allelic composition at SNP loci was determined by PCR-RFLP or tetra primer ARMS PCR.

Results. The TIMP-1 genotype TT in women and T in men at SNP +372 T/C was found to increase CD susceptibility (39% vs 23.8%, $P = 0.018$ and 67.9% vs 51.6%, $P = 0.055$, respectively), while women with this genotype were less prone to development of fistulae during follow-up (41.4% vs 68.3%, $P = 0.025$). Male IBD or CD patients carrying the TIMP-1 +372 T-allele expressed lower levels of TIMP-1 in surgically resected macroscopically inflamed tissue ($0.065 < P < 0.01$). The 5T5T genotype at MMP-3 SNP -1613 5T/6T increased the chance of stenotic complications in CD during follow-up (91.2% vs 71.8%, $P = 0.022$) but seemed to protect against colonic involvement of this disease at first endoscopic/radiologic examination (35.3% vs 59.5%, $P = 0.017$).

Conclusions. Allelic composition at the examined SNPs in genes coding for TIMP-1 and MMP-3 affect CD susceptibility and/or phenotype, i.e., fistulizing disease, stricture pathogenesis and first disease localization. These findings reinforce the important role of these proteins in IBD.

Introduction

Crohn's disease (CD) is characterized by chronic, patchy, transmural inflammation of the gastrointestinal tract, predominantly in the ileocecal area, while ulcerative colitis (UC) is manifested by chronic, continuous, rather superficial inflammation of the mucosal layers of the colon.[1,2] The incidence and prevalence of both CD and UC have increased in the Western population since the second World War,[3,4] and lately also increased in developing industrializing countries. Although there has been much controversy regarding etiology and pathogenesis of both forms of inflammatory bowel disease (IBD), recent evidence points to an exaggerated immune response to enteric bacterial flora in genetically susceptible individuals. Based on a higher disease concordance in monozygotic *vs* dizygotic twins,[5] a higher frequency of IBD in certain families and ethnic groups,[6,7] the association of IBD with genetic disorders like Turner's and Hermansky-Pudlak syndrome,[8,9] the presence of a genetic component in IBD is evident. Indeed, large-scale genome-wide linkage studies have mapped several regions of the human genome to IBD, i.e., 16q12 (IBD1),12q13 (IBD2), 6p21 (IBD3), 14q11 (IBD4), 19p13 (IBD5), 5q31-q33 (IBD6) and Xq21.3[10-15] and subsequent research has identified several CD predisposing mutations in the IBD1 gene encoding NOD2.[16] However, the different chromosomal locations found to be associated with IBD in these studies suggest disease heterogeneity: different sets of disease predisposing mutations may lead to a similar clinical outcome. This is corroborated by evidence obtained from animal models, where distinct genetic manipulations, for instance deletion of the DNA encoding TCR-α, IL-10 or TNF-α 3'UTR AU repeat motifs, all lead to ileitis and/or colitis.[17-19] Therefore, genes on other loci, not identified in the studies mentioned above, may also contribute to IBD susceptibility and worthy considering in this respect are the matrix metalloproteinases (MMPs) and their natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). The MMPs constitute a group of neutral, Ca- and Zn-activated endoproteinases and are involved in physiological matrix turnover during embryogenesis, angiogenesis, *etc.*[20] Production is tightly regulated at the transcriptional and post-transcriptional levels, and excessive MMP-mediated tissue destruction is prevented by strictly regulated activation mechanisms of the latent pro-enzyme and inhibition of the active

enzyme in a 1:1 stoichiometry by TIMPs. Recently, several functional single nucleotide polymorphisms (SNPs) in the genes encoding MMPs and TIMPs have been described. The insertion of an additional guanosine residue at -1607 in the promoter of MMP-1 creates a PEA3 consensus sequence next to an AP-1 binding site upregulating promoter activity, while the insertion of an additional thymidine at -1613 of the MMP-3 promoter results in decreased mRNA transcription.[21,22] The -1306 C/T transition in the promoter of MMP-2 results in decreased binding affinity for stimulating protein Sp1, leading to decreased mRNA transcription.[23] In contrast, the -1562 C/T transition in the promoter of MMP-9 results in the removal of a binding site for an unknown repressor protein, thus elevating transcription.[24] In TIMP-1 and -2 SNPs have been found in the exon part of the genes (+372 T/C and +303G/A, respectively). Although no effect on transcriptional activity/mRNA stability was observed, these SNPs might serve as markers in association studies.[25] Both MMP and TIMP expression are affected by TNF-α and this proinflammatory cytokine is known to play a pivotal role in IBD, particularly CD but also UC, as demonstrated by impressive clinical improvement following anti-TNF-α antibody infliximab administration.[26,27] The G/A transition at -308 in the TNF-α promoter might result in increased levels of circulating TNF-α protein,[28] thus inducing extra MMPs and/or TIMPs. Of note, the gene encoding TNF-α is mapped to the 6p21 IBD3 region, while the MMP and TIMP genes have not been mapped to any known IBD region (MMP-1, -3: 11q22-q23; MMP-2: 16q13; MMP-9: 20q11.2-q13.1; TIMP-1: Xp11.3-p11.23 and TIMP-2: 17q25). Conceivably, direct and indirect SNP-linked overproduction of MMPs and/or downregulation of TIMPs, would result in net destruction of tissue, impairment of intestinal barrier function, influx of bacteria and consequently excessive immune response, thus predisposing to or worsening IBD. Therefore, we analyzed the genotype distributions at these SNP loci of the genes encoding MMP-1, -2, -3, -9, TIMP-1, -2 and TNF-α in CD, UC and controls. Recently, we measured MMP and TIMP protein/activity levels in a large group of resected intestinal IBD tissues (Meijer *et al*, submitted) and here the expression data in a subgroup of which we also had DNA, are correlated to MMP, TIMP and TNF genotypes.

Materials and methods

Surgically resected intestinal mucosa from predominantly Dutch Caucasian patients with CD (*n* = 134, 40% male, median age at surgery 36.3 years, range 11.6-78.7 years) or UC [*n* = 111, 42% male, 37.8 (15.9-81.9) years], was collected in the period 1983-2002 at the department of Pathology, LUMC and stored at –70 ^{0}C. The control group consisted of 79 patients with colorectal carcinoma [CRC, macroscopically normal tissue obtained at least 10 cm away from evident neoplasia, 43% male, median age at surgery 56.4 (19.0-85.0) years] and 169 healthy volunteers [37% male, age at blood collection date 33.3 (18.2-72.9) years], recruited among spouses of patients from the outpatient clinic and through advertisement. Informed consent from participants and approval of the LUMC ethics committee for the study protocol was obtained.[29] In both the IBD and control groups, more than 95% of the participants were of Caucasian origin. Resected tissue was homogenized with a Turrax device, blood was centrifuged and genomic DNA was isolated using the salting out method[30] and reconstituted to 10 ng/µL in 0.01 mol/L Tris/0.1 mmol/L EDTA, pH = 7.5. Differential diagnosis of CD or UC was established by routine clinical, radiological and histological findings. Age at onset, localization at first endoscopy/radiology and development of fistulae and stenotic processes in a subset of CD patients (*n* =123) were recorded in medical files. The measurement of myeloperoxidase was according to the procedure described by Kruidenier *et al*,[31] while the MMP and TIMP protein/activity levels in IBD and CRC control tissue were measured previously by our group (Meijer *et al*.[32] and submitted). In brief, homogenates obtained from surgically resected tissue were appropriately diluted. The MMPs and TIMPs antigen levels were measured by ELISAs (MMP-2, -9, TIMP-1, -2) or by highly sensitive bio-immuno activity assays (BIA) involving the conversion of chromogenic peptide S-2444 by MMP-activated pro-urokinase (MMP-1,-3), with all BIAs performed in the presence of APMA to account for total MMP antigen levels. Allelic composition at the SNPs of interest was determined by PCR-RFLP (MMP-1, -3, -9, TIMP-1, -2) or tetra primer ARMS PCR (MMP-2), as described previously.[32,33] Differences between groups were assessed by Chi-square, Kruskal-Wallis or Mann-Whitney *U* tests, as indicated. Statistical significance was reached if two-tailed *P* value $\leqslant$ 0.05

Results

Allelic composition at SNP loci of MMP, TIMP and TNF genes

Between IBD and controls, no significant differences in genotype distribution were found at -1607 1G/2G and -1306 C/T of MMP-1 and -2 promoters, respectively (Table 1). Also, 1G MMP-1 (55.4 vs 51.4%) and C wild-type (75.5 vs 76.8%) MMP-2 allelic frequencies were similar in both groups. The MMP-3 and MMP-9 genotype distribution at -1613 5T/6T and -1562 C/T, respectively, were also similar. The TIMP-1 gene is located on the X-chromosome, thus the results are presented according to gender. In both men and women the T (T) genotype seems relatively abundant in IBD (men T 61.6 vs 51.6%; women TT 31.2 vs 23.8%) and especially in CD (men T 67.9 vs 51.6%, $P = 0.055$; women TT 39.0 vs 23.8%, $P = 0.018$, Table 2). No differences in genotype distribution were observed for TIMP-2 and TNF-α at +303 G/A and -308 G/A, respectively (Table 1). For all SNPs, genotype frequencies in the control group are similar to what was expected from the Hardy-Weinberg equilibrium, except for MMP-2 (CC, CT, TT: 61.9, 29.9, 8.2 observed vs 59.0, 35.7, 5.3% expected, $\chi^2 = 6.36$, $P < 0.05$). Genotype and allelic frequencies for all SNPs examined were similar in CD versus UC and also in the healthy volunteers vs the carcinoma controls. As MMPs and TIMPs are involved in cancer and metastasis, all analyses were repeated with a control group consisting only of the healthy volunteers ($n = 169$), yielding similar results as mentioned above.

Effect of MMP and TNF-α SNPs on CD phenotype

The median age at onset of disease in 123 CD patients with a full medical record was 21.5 (range 0.3-61.5) years. Patients stratified according to genotype at the SNPs examined had similar ages at onset (Table 3). At first endoscopic/radiologic examination, in 53.3% of the patients colonic w/wo ileal involvement was evident. The MMP-3 genotype was associated with disease localization ($P = 0.04$ for all three groups) and further analysis revealed a lower chance of colonic involvement at first endoscopy/radiology in patients with the 5T5T MMP-3 genotype ($P = 0.017$, 5T5T vs 5T6T and 6T6T combined). However, this genotype also conferred a major risk to development of stenotic complications: 91.2% of patients carrying the 5T5T genotype suffered from stenotic complications compared to 71.8% for the

Table 1. Genotype distributions at SNP loci in IBD patients compared to controls

Protein	SNP	Genotype	IBD (% of patients)	Controls (% of individuals)	Allele	IBD (% of total)	Controls (% of total)
MMP-1	-1607 1G/2G	1G1G	31	26.6	1G	55.4	51.4
	11q22-q23	1G2G	49	49.6	2G	44.6	48.6
		2G2G	20.1	23.8			
MMP-2	-1306 C/T	CC	57.7	61.9	C	75.5	76.8
	16q13	CT	35.6	29.9	T	24.5	23.2
		TT	6.7	8.2			
MMP-3	-1613 5T/6T	5T5T	29.2	27.9	5T	52.3	52
	11q23	5T6T	46.3	48.4	6T	47.7	48
		6T6T	24.6	23.8			
MMP-9	-1562 C/T	CC	72.4	69.7	C	85.6	84
	20q11.2-q13.1	CT + TT	27.6	30.3	T	14.4	16
♂, TIMP-1[1]	+372 T/C	T	61.6	51.6	T	61.6	51.6
	Xp11.3-p11.23	C	38.4	48.4	C	38.4	48.4
♀, TIMP-1[1]	+372 T/C	TT	31.2	23.8	T	52.8	50.7
	Xp11.3-p11.23	TC	43.3	53.6	C	47.2	49.3
		CC	25.5	22.5			
TIMP-2	+303 G/A	GG	77.5	78.7	G	88.3	89.1
	17q25	GA + AA	22.5	21.3	A	11.7	10.9
TNF-α	-308 G/A	GG	68.8	69.7	G	82.0	83.0
	6p21	GA + AA	31.4	30.3	A	18.0	17.0

n = 239-240/245 IBD patients *vs* *n* = 244/248 controls with successful genotype determinations. [1]For TIMP-1, ♂ these numbers are 99/101 *vs* 93/96 and for TIMP-1, ♀ 141/144 *vs* 151/152. Note: for MMP-9, TNF-α and TIMP-2 frequency of the homozygote mutant genotype (TT, AA and AA, respectively), was below 5% and these groups were combined with the corresponding heterozygote group, solely for the purpose of accurate statistical analysis. No statistically significant differences (Chi-Square testing) in genotype distributions or allele frequencies were found between IBD and controls.

Table 2. TIMP-1 genotype distribution at SNP + 372 C/T in CD patients compared to controls

Protein	Geno-type	CD (% of patients)	Controls (% of individuals	*P* [1]	Allele	CD (% of total)	Controls (% of total)	*P*-value
TIMP-1, ♂	T	67.9	51.6	0.055	T	67.9	51.6	0.055
	C	32.1	48.4		C	32.1	48.4	
TIMP-1, ♀	TT	39	23.8	0.018	T	56.5	50.7	0.238
	TC	35.1	53.6		C	43.5	49.3	
	CC	26	22.5					

♂: *n* = 53/54 *vs* 93/96; ♀: *n* = 77/80 *vs* 151/152 successful genotype determinations, respectively. [1]*P*-value, Chi-Square test.

other genotypes (*P* = 0.022). The allelic polymorphisms at other SNP loci were not associated with disease localization or stricture involvement. Of all CD patients, 80/123 or 65.0% developed peri-anal, entero-entero or entero-cutaneous fistulae during follow-up. Female patients with the TT genotype at +372 SNP of TIMP-1 appeared less prone to develop fistulae (*P* = 0.08), and when the TT group was compared with the combined TC and CC group, this result was statistically significant (*P* = 0.025).

Effect of SNPs on protein expression

Male IBD patients carrying the T allele at SNP +372 expressed lower levels of TIMP-1 in inflamed tissue compared to those carrying the C allele, *P* = 0.009 (Fig. 1), with similar MPO levels in both groups [median 24.2 (range 9.1-80.4) vs 28.6 (2.5-75.9) U/g, P = 0.194]. In male CD patients a similar pattern in TIMP-1 expression was observed [6.8 (1.7-18.6) vs 9.2 (1.8-19.9) ng/mg TIMP-1, *n* = 46 vs 19, T vs C allele, respectively], although not longer statistically significant (*P* = 0.065). However, female IBD or CD patients carrying the TT, TC or CC genotype expressed similar levels of TIMP-1 in inflamed tissue. The respective protein expression was not affected by genotype at other MMP and TIMP SNPs in inflamed intestinal tissue. In non-inflamed IBD and control CRC tissue, no

Table 3. Effect of MMP, TIMP and TNF-α allelic SNP composition on age at onset of disease, colon involvement at first endoscopic/radiologic examination and development of fistulae or stenotic strictures during follow-up [median 24.7 (range 3.3-58.5) yr] in CD

Protein	SNP	Genotype	Number of patients	Age (yr) at onset of disease, median (range)	Colonic involvement at first examination [1]	Fistulae during FU [1]	Strictures during FU [1]
MMP-1	-1607 1G/2G	1G1G	38	20.7 (0.3-45.6)	52.6	71.1	84.2
		1G2G	54	22.4 (7.0-61.5)	50	63	75.9
		2G2G	26-27	21.0 (3.4-40.2)	57.7	63	70.4
MMP-2	-1306 C/T	CC	66-67	20.1 (5.8-61.5)	57.6	64.2	74.6
		CT+TT	52 [2]	22.4 (0.3-50.1)	46.2	67.3	80.8
MMP-3	-1613 5T/6T	5T5T	34	22.4 (0.3-53.6)	35.3 [a]	70.6	91.2 [a]
		5T6T	54	19.3 (3.4-61.5)	63	66.7	74.1
		6T6T	30-31	23.5 (7.2-49.1)	53.3	58.1	67.7
MMP-9	-1562 C/T	CC	88-89	21.0 (0.3-61.5)	53.4	68.5	75.3
		CT + TT	30 [2]	22.7 (7.2-45.5)	50	56.7	83.3
♂, TIMP-1	+372 T/C	T	32	18.9 (0.3-48.3)	53.1	78.1	87.5
		C	17	18.5 (5.9-61.5)	52.9	76.5	94.1
♀, TIMP-1	+372 T/C	TT	29	24.3 (7.0-56.8)	55.2	41.4 [c]	69.0
		TC	22-23	23.5 (3.4-39.6)	45.5	69.6	78.3
		CC	18	22.2 (10.4-49.1)	55.6	66.7	55.6
TIMP-2	+303 G/A	GG	91-92	21.5 (0.3-61.5)	56.0	68.5	77.2
		GA + AA	27 [2]	22.4 (3.4-56.8)	40.7	55.6	77.8
TNF-α	-308 G/A	GG	79-80	22.6 (0.3-61.5)	50.6	68.8	78.8
		GA + AA	39 [2]	18.9 (3.4-45.5)	56.4	59.0	74.4

[1] Expressed as (% of patients); [2] Only 7, 1, 2 and 4 patients were carrying the MMP-2, -9, TIMP-2 or TNF-α mutant genotype, respectively, and were combined with the heterozygote group for statistical purposes. Differences in phenotype between genotypes were tested for statistical significance by Kruskal-Wallis/Mann-Whitney *U* (onset) or Chi-Square test (colon involvement/fistulae and stricture development); [a] $P \leqslant 0.03$ *vs* 5T6T and 6T6T combined; [c] $P = 0.025$ *vs* TC and CC combined.

differences in protein levels were observed between patients stratified to genotype. Finally, allelic composition at TNF-α-308 G/A was not associated with higher or lower levels of MMPs or TIMPs in inflamed and non-inflamed IBD or control tissue.

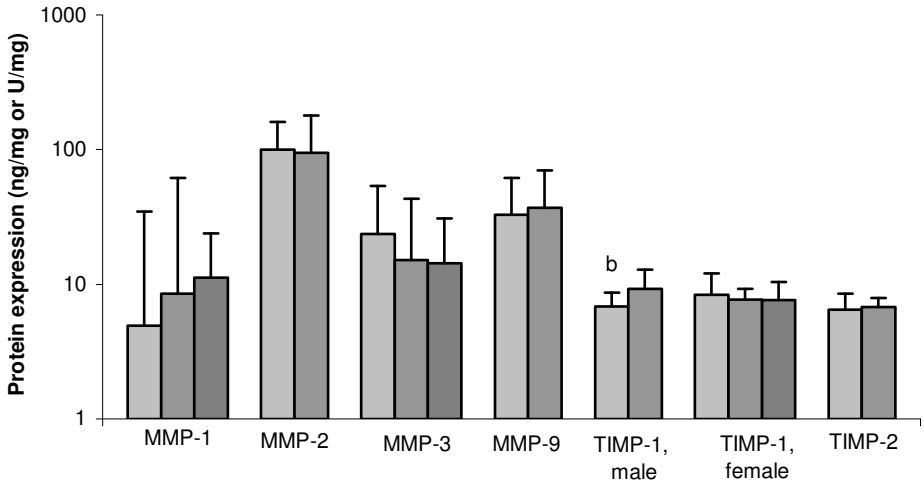

Figure 1. Protein expression in inflamed IBD tissue stratified to genotype. Values represent the median + 75th percentile and are in ng/mg (MMP-2, -9, TIMP-1, -2) or in arbitrary units/mg (MMP-1, -3). From left to right; MMP-1: 1G1G, 1G2G, 2G2G (*n* = 6, 17, 5); MMP-2: CC, CT + TT (*n* = 99, 77); MMP-3: 5T5T, 5T6T, 6T6T (*n* = 21, 33, 16); MMP-9: CC, CT + TT (*n* = 129, 46); TIMP-1 male: T, C (*n* = 47, 24); TIMP-1 female: TT, TC, CC (*n* = 42, 37, 26); TIMP-2: GG, GA + AA (*n* = 135, 41). [b] $P < 0.01$, Mann-Whitney *U* test.

Discussion

In this study we found increased susceptibility to CD in men and women carrying the T and TT genotype, respectively, at TIMP-1 SNP +372. The X chromosome region p11.3-p11.23 might thus represent a novel linkage marker in IBD, extending the results obtained in previous genome-wide linkage studies.[14,34] Women with this genotype also appear less prone to the development of fistulae. The direct or indirect involvement of the X-chromosome in CD etiopathogenesis is further corroborated by a higher incidence of CD in women compared to men,[35] the

association of CD with X-linked Turner's syndrome[8] and the higher incidence of extra-intestinal complications and surgery recurrence rates in female compared to male CD patients.[36] Importantly, in men the T allele at SNP +372 was accompanied with a lower TIMP-1 protein expression in inflamed tissue. The lower TIMP-1 protein levels relative to MMP in susceptible individuals might shift the balance to a more proteolytic mucosal Crohn's disease phenotype. The TIMP-1 SNP might also be linked to other markers on the X-chromosome increasing CD susceptibility and conferring protection against fistulae pathogenesis thus explaining the observed results in women. We observed no association between allelic composition at MMP-3 SNP-1613 and susceptibility to IBD. Our findings in UC confirm previous publications on primary sclerosing cholangitis and UC,[22,37] but those on CD are different from the results obtained by the group of Pender *et al*,[38] who noted increased susceptibility to sporadic, but not familial CD in individuals carrying the 5T allele. These contrasting results might arise from a different proportion of sporadic versus familial cases in our study. We also found a decreased chance of colonic involvement at first endoscopic/radiologic examination and a higher incidence of stenotic complications in patients carrying the 5T5T MMP-3 genotype at SNP -1613. Previously, over transmission of the 5T allele was associated with ileal localisation and stenosis in CD CARD15 mutation carriers[38] and the group of Warnaar *et al*.[39] reported increased levels of MMP-3 in stenotic and pre-stenotic resected CD ileum, pointing to an MMP-3 mediated altered clinical course of CD patients by an, as yet, unidentified mechanism. The 5T5T genotype was reported to both increase[40,41] and decrease[42] MMP-3 protein expression, but in our study patients stratified according to MMP-3 genotype expressed similar MMP-3 total activity. Previously, the A allele at TNF-α SNP -308 was reported to increase susceptibility to UC,[43] CD[44] and the incidence of fistulae in CD,[45] possibly mediated by an increased promoter activity.[46,47] In contrast, we found no effect of allelic composition at this SNP on disease risk and phenotype, in line with other reports,[48,49] adding further complexity to this matter. As mentioned before, the patient populations might differ dependent on the genetic (ethnic) background, thus explaining the contrasting results. We could not demonstrate an association of MMP-1, -2, -9 and TIMP-2 SNPs with disease susceptibility or clinical course of

disease, in line with previous (genome-wide) linkage reports.[11-13,37,50] As other studies have clearly shown the involvement of these proteins in IBD pathology,[51,52] it seems that they primarily function as mediators/effectors instead of initiators during IBD etiopathogenesis. However, the regulation of these proteins by immunosuppressive medication, such as infliximab, might be dependent on the allelic composition at the SNPs examined, as previously shown by *ex vivo* explant studies from our group.[32] In principle, enhanced MMP expression might also be associated with SNPs in other genes, for instance with those encoding cytokines regulating MMP expression, e.g., IL-1β[44] and TNF-α. Dependent on the presence of relevant cis-acting elements in the promoter sequence, especially MMP-1 and MMP-9 would be affected,[53,54] but we found no effect. In summary, several studies reported associations between SNPs in diverse genes and IBD.[16,48, 55-59] We have focused on the SNPs in genes coding for matrix remodeling proteins, i.e., MMPs and TIMPs, and believe the T allele at SNP +372 T/C in TIMP-1 might be involved in CD susceptibility in both sexes and in men by down-regulating TIMP-1 expression, while the 5T5T genotype at MMP-3 -1613 might protect for colonic disease localization but also confers a major risk to stenotic complications. These findings reinforce the potential role of MMP and TIMPs in IBD and should be confirmed in larger prospective follow-up studies.

Acknowledgments

The technical assistance of M. van den Berg, J.J. van der Reijden and W. van Duijn is greatly appreciated.

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Chapter 7

Matrix Metalloproteinases and their Tissue Inhibitors as Prognostic Indicators for Diagnostic and Surgical Recurrence in Crohn's Disease

Short title: Recurrence in CD

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This Study was published in Inflamm Bowel Dis 2009; 15(1): 84-92

Abstract

Background/aims. Recurrence of disease after surgically induced remission constitutes a major and largely unpredictable problem in Crohn's disease (CD). Matrix metalloproteinases (MMP) and the tissue inhibitors of metalloproteinases (TIMP) are involved in the (etio)pathogenesis of CD and may thereby also affect postsurgical outcome. We studied the predictive value of 1) allelic composition at MMP, TIMP, and TNF-α single nucleotide polymorphism loci, and 2) MMP and TIMP intestinal protein levels relative to important clinical variables for recurrence of CD after resection of diseased bowel.

Methods. From 87 CD patients with a full medical record, surgically resected tissue was homogenized and analyzed for single nucleotide polymorphism (SNP) genotype and MMP-TIMP protein levels. The prognostic value of these parameters was determined using the uni- and multivariate Cox proportional hazards analyses.

Results. The T-allele at TIMP-1 SNP +372 T/C was found to be associated with an increased risk for surgical recurrence. Higher levels of TIMP-1, TIMP-2, and MMP-9 in noninflamed CD tissue, but not in inflamed tissue, and negative smoking status independently protected against diagnostic and/or surgical recurrence.

Conclusions. The TIMP-1 SNP +372 T allele with an increased risk of recurrence is in line with our previous results demonstrating increased CD susceptibility and low TIMP-1 protein expression associated with this allele. High TIMP and MMP-9 levels in non-inflamed tissue are predictive of a favorable disease recurrence in CD. The contribution of MMP-9 and TIMPs to disease recurrence appears not to be mediated by smoking status, since no correlation with this parameter could be demonstrated.

Introduction

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract. Although the etiology is largely unknown, current understanding suggests that the sustained immune infiltration manifests as an aberrant reaction to luminal bacteria in genetically susceptible individuals.[1] It is characterized by chronic, segmental, and transmural inflammation of the bowel wall, particularly ileocolonic, treated with immunomodulating drugs to induce clinical remission. Despite advances in medical therapy, still a large proportion of CD patients will eventually require resection of diseased bowel, with an estimated cumulative probability during the first decade after diagnosis ranging between 30% and 60%.[2,3] One year after resection, endoscopic and clinical recurrence occurs in 70% and 20% of patients, respectively.[4] The cumulative second surgical recurrence rate amounts to 35.9% 10 years after first bowel resection, with a small but substantial group at hazard for even more subsequent resections (third surgical recurrence rate 39.8% 5 years after the second bowel resection).[5] Several studies focused on identifying predictive variables for postsurgical recurrence, including age at onset of disease, age at time of surgery, duration of disease before initial operation, type of surgery (i.e., laparoscopic versus open conventional), disease localization, gender, histology, etc., with sometimes inconclusive or even contrasting results.[6-14] Smoking behavior, however, was consistently found to adversely affect disease prognosis, both in medical and surgically induced remission.[15] Identification and selection of patients prone to recurrence would enhance the effectiveness of adjuvant medical therapy with azathioprine, ornidazole, or infliximab,[16-18] thus limiting costs and drug-related side effects.

Matrix metalloproteinases (MMP) constitute a group of neutral endoproteinases, collectively capable of cleaving various intestinal matrix proteins, with proteolytic activity tightly regulated by their natural inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).[19] In inflamed CD and ulcerative colitis tissue, several MMPs, including MMP-1, -3, -9, and -12 relative to TIMP-1 and -2 are overexpressed[20-27] leading to an increase in net MMP proteolytic activity.[28,29] Even in the macroscopically normal proximal resection margin, an upregulation of MMP-3 has been observed.[30] Part of this proteolytic phenotype seen in

inflammatory bowel diseases (IBDs) may be attributed to increased TNF-α signaling.[31,32] In the genes coding for MMPs, TIMPs, and TNF-α, single nucleotide polymorphisms (SNPs) have been described, and allelic composition at these SNPs may determine protein expression. For instance, MMP-1 protein expression is upregulated in cells carrying 2 copies of the 2G allele at MMP-1 promoter –1607.[33] In previous reports, the 5T and T allele at SNP MMP-3 –1613 5T/6T and TIMP-1 +372 T/C, respectively, were found to be associated with CD,[34,35] while contradictory results were obtained for the –308 G/A SNP at the TNF-α gene.[36,37] Therefore, CD patients with different genetic MMP, TIMP, or TNF-α constitution may express different levels of corresponding proteins and follow a different clinical course after surgically induced remission. Previously, we determined allelic composition at MMP-1, -2, -3, -9, TIMP-1, -2, and TNF-α SNP loci in CD patients and also measured MMP and TIMP protein levels in surgically resected inflamed and macroscopically normal tissue.[29,34] Here we relate the CD SNP allelic distribution and tissue protein expression to postsurgical recurrence data in a multivariate model including major clinical variables.

Materials and methods

Patient population

For this retrospective study, 87 predominantly Dutch Caucasian CD patients with a full medical record and prospectively collected resection specimens were included. Diagnosis of CD was established by routine endoscopical, radiological, and histological examination. Surgical intestinal resections with anastomosis of macroscopically normal bowel were performed in the period 1984–2000, median follow-up after resection was 8.4 (0.9–19.1) years. Location of disease was often ileocecal (n = 66), with 8 and 13 patients undergoing small bowel resection or (sub)total colectomy, respectively. Indication for surgery was stenotic narrowed tissue, medical refractory disease, or perforation (n = 36, 50, and 1, respectively). Diagnostic recurrence was defined as a rise in clinical symptoms with endoscopy and/or radiology-confirmed inflammation of previously unaffected bowel. Surgical recurrence was defined as resection of diseased tissue macroscopically normal at

previous surgery.

Genotype and protein determination

Resected tissue was collected at the Department of Pathology, LUMC, and stored at -70°C until analysis. Mucosa was homogenized with a Turrax device, genomic DNA was isolated according to the salting out method[38] and reconstituted to 10 ng/µL in 0.01 M Tris / 0.1 mM EDTA, pH 7.5. Allelic composition at the SNP loci studied was determined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) (MMP-1, -3, -9, TIMP-1, -2, TNF-α) or tetra primer ARMS PCR (MMP-2), as described previously.[39,40] In 1 patient DNA isolation failed and the genotype could not be determined. From 85 and 76 patients, respectively, inflamed and noninflamed mucosa was obtained and 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). Antigen protein levels of MMP-2 and MMP-9 were measured in appropriately diluted homogenates by our in-house enzyme-linked immunosorbent assays (ELISAs), as described before,[41] while TIMP-1 and -2 protein were measured by commercially available ELISAs from R&D Systems (Minneapolis, MN), according to the manufacturer's instructions. All antigen levels were corrected for total protein amount, as determined by the method of Lowry *et al*.[42] The protein levels of MMP-1, MMP-3, and TNF-α were either not assessed or at a too low number for adequate analyses.

Statistical analysis

Differences between subgroups were calculated using the Mann–Whitney U-test or Pearson Chi Square test as indicated. For disease follow-up analyses the clinical parameters were dichotomized as follows: smoking habit after surgical resection (yes versus no), gender (male versus female), medication before surgical resection (mild: nothing or mesalazine versus strong: corticosteroids and/or immunomodulatory drugs w/wo mesalazine), age at surgery (≤ 27.6 versus >27.6 years), age at onset of disease (≤ 16.65 years versus >16.65 years), number of former resections (0 versus >0), and time lag between onset of disease and surgical resection (≤ 11.89 versus >11.89 years). Cutoff values for continuous

clinical variables, MMP, and TIMP levels were optimized using receiver operator characteristic (ROC) analyses. Multivariate analyses were performed with the Cox proportional hazards method by separately adding variables to the dichotomized clinical parameters. Survival curves were constructed using the method of Kaplan and Meier including log-rank tests. Differences were considered significant when $P < 0.05$. Analyses were performed using the SPSS statistical package (Release 11.0, Chicago, IL).

Results

Clinical parameters and recurrence

In the univariate Cox analyses the 49 patients with a positive smoking status were at increased risk for developing diagnostic recurrence compared to the 38 non-smokers (hazard ratio [HR] 1.846, $P < 0.05$; Table 1). Median time of reaching the diagnostic recurrence endpoint dropped from 8.2 to 2.8 years ($P = 0.02$; Fig. 1A). Young age at surgery and at onset of disease (27.6 and 16.65 years, respectively) also exposed patients to earlier diagnostic recurrence. Gender, medication 1 month prior to surgery, number of previous resections, and time lag between onset of disease and surgery had no effect. In multivariate analyses including all clinical variables, only smoking maintained its prognostic value (HR 2.002, $P < 0.02$). Smoking, but not the other clinical parameters, also significantly increased the risk for surgical recurrence (HR 4.033, $P = 0.001$), with the median time lag for re-resection dropping from 17.2 to 9.3 years ($P = 0.0005$; Fig. 1B) and proved an independent prognostic indicator with a similar hazard ratio in the multivariate analyses. However, smoking was not associated with MMP-TIMP genotype or tissue protein levels (Suppl. Table X), providing a rationale for further hazard analyses including these parameters.

Genotype and tissue MMP-TIMP protein levels versus recurrence

The allelic distribution at MMP, TIMP, and TNF-α SNP loci was not associated with diagnostic recurrence (Table 2). In univariate analyses, only the C allele at SNP TIMP-1 +372 T/C was found to be protective against surgical recurrence. Patients

Table 1. Cox regression analyses of selected clinical variables in relation to diagnostic (A) and surgical (B) recurrence after resection of diseased bowel in 87 CD Patients.

A) Diagnostic recurrence

		Univariate			Multivariate		
Parameter	*N*	HR	95% CI	*P*	HR	95% CI	*P*
Smoking			1.091-				
Negative vs positive	38/49	1.846	3.124	0.022	2.002	1.144-3.504	0.015
Gender			0.857-				
Male vs female	35/52	1.445	2.436	NS	1.302	0.721-2.351	NS
Medication*			0.858-				
Mild vs strong	38/49	1.441	2.419	NS	1.146	0.626-2.100	NS
Age at surgery			0.315-				
≤ 27.6 vs > 27.6 yrs	25/62	0.534	0.904	0.020	0.648	0.298-1.409	NS
Age at onset of disease			0.313-				
≤ 16.65 vs > 16.65 yrs	22/65	0.537	0.923	0.024	0.587	0.300-1.150	NS
Nr of previous resections			0.583-				
0 vs >0	52/35	0.975	1.630	NS	1.443	0.774-2.689	NS
Time lag onset of							
disease-resection			0.510-				
≤ vs > median (11.89 yrs)	44/43	0.847	1.409	NS	0.874	0.459-1.664	NS

B) Surgical recurrence

		Univariate			Multivariate		
Parameter	*N*	HR	95% CI	*P*	HR	95% CI	*P*
Smoking			1.733-			1.720-	
Negative vs positive	38/49	4.033	9.385	0.001	4.206	10.286	0.002
Gender			0.894-				
Male vs female	35/52	1.906	4.063	NS	1.664	0.730-3.792	NS
Medication*			0.592-				
Mild vs strong	38/49	1.202	2.438	NS	0.896	0.380-2.115	NS
Age at surgery			0.442-				
≤ 27.6 vs > 27.6 yrs	25/62	0.938	1.989	NS	1.056	0.353-3.156	NS
Age at onset of disease			0.630-				
≤ 16.65 vs > 16.65 yrs	22/65	1.532	3.727	NS	1.256	0.451-3.503	NS
Nr of previous resections			0.459-				
0 vs >0	52/35	0.932	1.894	NS	1.457	0.605-3.512	NS
Time lag onset of							
disease-resection			0.462-				
≤ vs > median (11.89 yrs)	44/43	0.926	1.855	NS	0.719	0.267-1.935	NS

* Medication: mild = nothing or mesalazine, strong = corticosteroids, immunomodulatory drugs w/wo mesalazine one month prior to surgical resection. *N*, number of patients; HR, hazard ratio; CI, confidence interval; *P*, statistical significance.

Table 2. Cox proportional hazard analysis for 86 CD Patients testing allelic distribution at MMP, TIMP and TNF-α SNP loci versus clinical parameters in relation to diagnostic (A) and surgical (B) recurrence after resection of diseased bowel.

A) Diagnostic recurrence

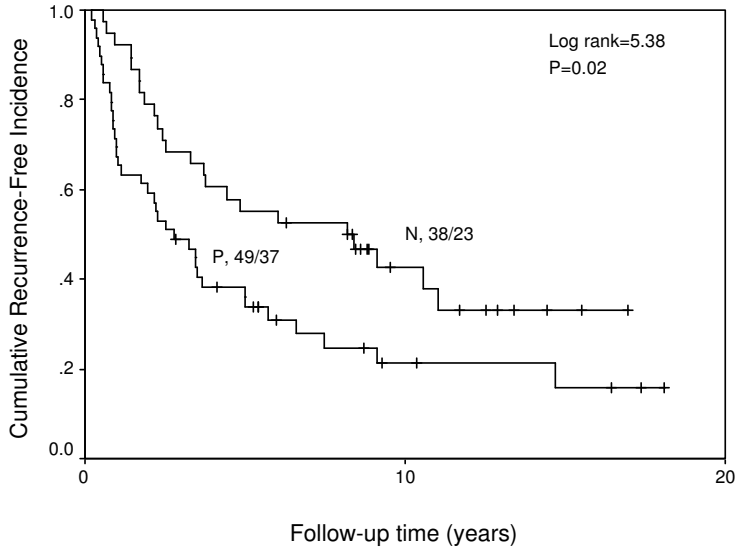
		Univariate			Multivariate		
Parameter	*N*	HR	95% CI	*P*	HR	95% CI	*P*
MMP-1 SNP –1607 1G/2G 1G1G vs 1G2G + 2G2G	26/40+20	1.252	0.712-2.201	NS	1.282	0.699-2.352	NS
MMP-2 SNP –1306 C/T CC vs CT + TT	46/35+5	0.841	0.502-1.407	NS	0.608	0.349-1.061	NS
MMP-3 SNP –1613 5T/6T 5T5T vs 5T6T + 6T6T	24/40+22	0.980	0.557-1.724	NS	0.946	0.504-1.778	NS
MMP-9 SNP –1562 C/T CC vs CT + TT	63/23+0	1.261	0.717-2.218	NS	1.324	0.712-2.461	NS
TNF-α SNP –308 G/A GG vs GA + AA	61/21+4	0.863	0.485-1.537	NS	0.636	0.336-1.205	NS
TIMP-1 SNP +372 T/C T(T)+CT vs C(C)	41+19/26	0.959	0.548-1.679	NS	1.224	0.662-2.265	NS
TIMP-2 SNP +303 G/A GG vs GA + AA	65/20+1	1.251	0.701-2.233	NS	1.026	0.562-1.874	NS

B) Surgical recurrence

		Univariate			Multivariate		
Parameter	*N*	HR	95% CI	*P*	HR	95% I	*P*
MMP-1 SNP –1607 1G/2G 1G1G vs 1G2G + 2G2G	26/40+20	0.784	0.379-1.619	NS	0.925	0.423-2.022	NS
MMP-2 SNP –1306 C/T CC vs CT + TT	46/35+5	1.177	0.587-2.364	NS	0.996	0.485-2.045	NS
MMP-3 SNP –1613 5T/6T 5T5T vs 5T6T + 6T6T	24/40+22	0.698	0.340-1.433	NS	1.064	0.456-2.481	NS
MMP-9 SNP –1562 C/T CC vs CT + TT	63/23+0	0.801	0.360-1.785	NS	0.785	0.338-1.824	NS
TNF-α SNP –308 G/A GG vs GA + AA	61/21+4	0.575	0.248-1.333	NS	0.471	0.192-1.156	NS
TIMP-1 SNP +372 T/C T(T)+CT vs C(C)	41+19/26	0.391	0.160-0.955	0.039	0.385	0.143-1.038	NS (*P* = 0.059)
TIMP-2 SNP +303 G/A GG vs GA + AA	65/20+1	1.868	0.876-3.985	NS	1.558	0.685-3.545	NS

N, number of patients; HR, hazard ratio; CI, confidence interval; *P*, statistical significance.

A Diagnostic recurrence smoking



B Surgical recurrence smoking

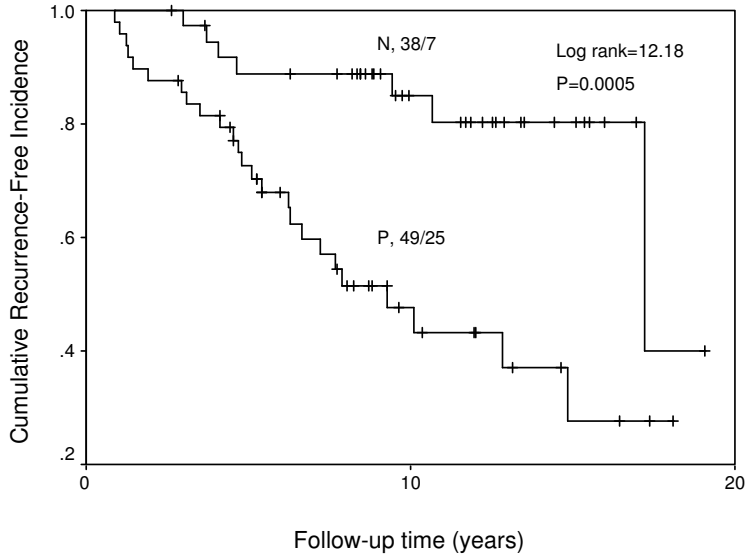


Figure 1. Kaplan–Meier recurrence-free incidence analysis for smoking status. Positive smoking status increased the risk for diagnostic (A) and surgical (B) recurrence, dropping median duration between surgery and diagnostic recurrence from 8.2 to 2.8 years and between surgery and re-resection from 17.2 to 9.3 years, $P < 0.05$. N, P = negative or positive smoking status after surgery, respectively. X/Y = number of patients / number of patients with recurrence.

carrying the T(T) or CT compared to C(C) genotype underwent re-resection 10.7 versus 19.1 years after initial surgery ($P < 0.05$; Fig. 2A). In the univariate analysis we found HR 0.391 ($P < 0.04$) and this association was borderline non-significant in the multivariate analysis ($P = 0.059$). The TIMP-1 gene is X-linked, a survival analysis stratified to gender yielded a similar trend for women (data not shown). High levels of TIMP-1, TIMP-2, and remarkably MMP-9 (2.94, 4.66 and 5.11 ng/mg, respectively) in noninflamed tissue decreased the risk for diagnostic recurrence (Table 3). Of these only MMP-9 was found to be an independent prognostic variable (HR = 0.498, $P < 0.05$). High levels of TIMP-1, TIMP-2, and MMP-9 (2.88, 4.66, and 5.11 ng/mg, respectively) in noninflamed tissue were also found to be protective against surgical recurrence. For instance, the median time lag between surgery and re-resection was 10.7 years in 17 patients with less than or equal to 2.88 ng/mg TIMP-1 in noninflamed tissue while in 59 patients with TIMP-1 levels above this threshold it was 17.2 years ($P = 0.03$; Fig. 2B). A similar pattern was observed for MMP-9 (Fig. 2C). These parameters were also statistically significant in the multivariate analysis. However, levels of MMP-2 in noninflamed tissue and of MMP-2, MMP-9, TIMP-1, and TIMP-2 in inflamed tissue were not relevant to the risk for diagnostic or surgical recurrence (Table 3) and neither were the MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios (data not shown).

Discussion

In our relatively small cohort of 87 Dutch CD patients the T allele at SNP TIMP-1 372 T/C was found to increase the risk for surgical recurrence, although this effect just missed statistical significance in multivariate analysis. Patients carrying the T(T) or CT compared to C(C) genotype underwent re-resection much earlier (10.7 versus 19.1 years) after initial surgery. The TIMP-1 genotype had no effect on diagnostic recurrence, indicating a late onset or relatively mild influence of this polymorphism on CD progression. We also observed an increased risk on surgical (and diagnostic) recurrence in patients with a low TIMP-1 protein level in noninflamed intestinal tissue. Patients with TIMP-1 ≤ 2.88 ng/mg underwent re-resection after 10.7 years, almost 7 years earlier compared to patients with TIMP-1 levels above this value. We previously reported increased CD susceptibility and low TIMP-1 levels associated with the TIMP-1 T allele.[34] Combined with our current data, we postulate that this allele downregulates TIMP-1 expression by an as yet

Table 3. Cox proportional hazard analysis for testing MMP and TIMP protein levels in noninflamed and inflamed CD Tissue (*n* = 76 and 85, respectively) versus clinical parameters in relation to diagnostic (A) and surgical (B) recurrence after resection of diseased bowel.

A) Diagnostic Recurrence

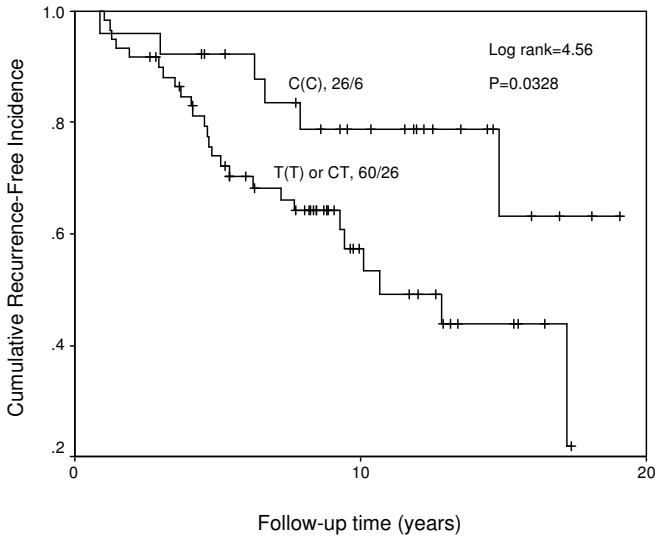
		Univariate			Multivariate		
Parameter	*N*	HR	95% CI	*P*	HR	95% CI	*P*
MMP-2 non-inflamed	38/		0.490-				
Median (60.23 ng/mg)	38	0.839	1.436	NS	1.024	0.529-1.982	NS
MMP-9 non-inflamed ≤ 5.11	16/		0.218-				
vs > 5.11 ng/mg	60	0.404	0.748	0.004	0.498	0.252-0.984	0.045
TIMP-1 non-inflamed ≤ 2.94	19/		0.287-				
vs > 2.94 ng/mg	57	0.509	0.902	0.021	0.658	0.350-1.237	NS
TIMP-2 non-inflamed ≤ 4.66	12/		0.263-				
vs > 4.66 ng/mg	64	0.513	1.003	0.051	0.661	0.313-1.396	NS
MMP-2 inflamed Median	43/		0.467-				
(110.85 ng/mg)	42	0.783	1.313	NS	0.701	0.387-1.269	NS
MMP-9 inflamed Median	43/		0.613-				
(37.63 ng/mg)	42	1.027	1.720	NS	1.134	0.664-1.938	NS
TIMP-1 inflamed Median	43/		0.550-				
(8.31 ng/mg)	42	0.923	1.548	NS	0.941	0.541-1.636	NS
TIMP-2 inflamed Median	43/		0.603-				
(6.9 ng/mg)	42	1.012	1.697	NS	1.385	0.789-2.432	NS

B) Surgical Recurrence

		Univariate			Multivariate		
Parameter	*N*	HR	95% CI	*P*	HR	95% CI	*P*
MMP-2 non-inflamed	38/		0.546-				
Median (60.23 ng/mg)	38	1.140	2.378	NS	1.226	0.483-3.116	NS
MMP-9 non-inflamed ≤ 5.11	16/		0.131-				
vs > 5.11 ng/mg	60	0.280	0.601	0.001	0.279	0.114-0.686	0.005
TIMP-1 non-inflamed ≤ 2.88	17/		0.200-				
vs > 2.88 ng/mg	59	0.434	0.942	0.035	0.403	0.174-0.932	0.034
TIMP-2 non-inflamed ≤ 4.66	12/		0.142-				
vs > 4.66 ng/mg	64	0.326	0.751	0.008	0.328	0.126-0.856	0.022
MMP-2 inflamed Median	43/		0.546-				
(110.85 ng/mg)	42	1.107	2.247	NS	0.979	0.450-2.130	NS
MMP-9 inflamed Median	43/		0.727-				
(37.63 ng/mg)	42	1.492	3.059	NS	1.772	0.845-3.716	NS
TIMP-1 inflamed Median	43/		0.535-				
(8.31 ng/mg)	42	1.084	2.198	NS	0.926	0.437-1.960	NS
TIMP-2 inflamed Median	43/		0.428-				
(6.9 ng/mg)	42	0.874	1.784	NS	0.998	0.471-2.114	NS

N, number of patients; HR, hazard ratio; CI, confidence interval; P, statistical significance

A Surgical recurrence TIMP-1 genotype



B Surgical recurrence TIMP-1 level

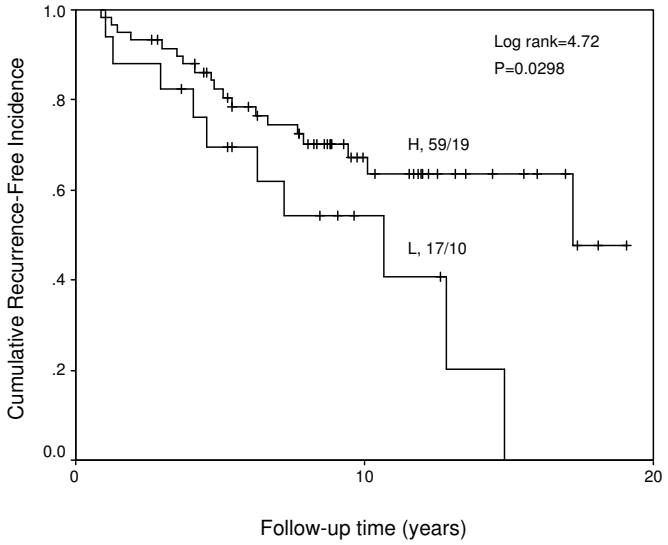


Figure 2. Kaplan–Meier analysis illustrating surgical recurrence-free incidence curves stratified by TIMP-1 SNP + 372 T/C (A), TIMP-1 level (B), and MMP-9 level (C, next page) in non-inflamed CD tissue

C Surgical recurrence MMP-9 level

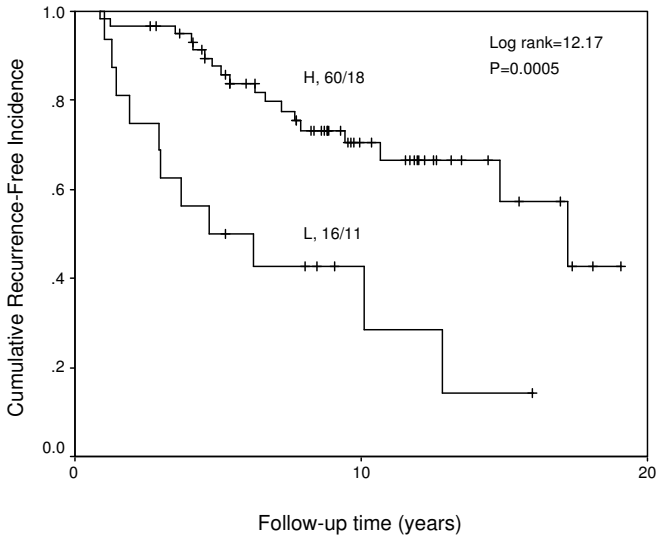


Figure 2, continued. Kaplan–Meier analysis illustrating surgical recurrence-free incidence curves stratified by TIMP-1 SNP + 372 T/C (A), TIMP-1 level (B), and MMP-9 level (C) in noninflamed CD tissue. Patients carrying the T(T) or CT compared to C(C) genotype underwent re-resection 10.7 versus 19.1 years after initial surgery ($P < 0.05$). High levels of both proteins were protective against surgical recurrence and increased median duration to re-resection from 10.7 (TIMP-1) or 4.7 (MMP-9) to 17.2 years in both, $P < 0.03$. H, L = high and low levels in noninflamed tissue (> and ≤ 2.88 [TIMP-1] versus > and ≤ 5.11 [MMP-9] ng/mg). X/Y = number of patients / number of patients with recurrence.

undefined mechanism, shifting the MMP/TIMP balance to a more proteolytic phenotype, enhancing CD susceptibility and worsening CD (postsurgical) prognosis. Alternatively, the TIMP-1 SNP is in linkage disequilibrium with another predisposing locus. The 372 SNP has been studied in other diseases with possible involvement of TIMP-1 in the pathogenesis, i.e., systemic sclerosis, intracranial and abdominal aneurysms, with inconclusive results.[43-45] CD patients with TIMP-2 levels >4.66 ng/mg in noninflamed tissue were also protected from developing surgical recurrence, presumably acting via a similar mechanism as described for TIMP-1. As TIMP-1/MMP-9 and TIMP-2/MMP-2 ratios were not associated with recurrence-free intervals, it appears that both TIMPs might exert their protective

effect through inhibition of other metalloproteinases, for instance MMP-3, -7, -12, or -13.[46-48] Remarkably, MMP-9 levels >5.11 ng/mg in non-inflamed tissue decreased the risk for diagnostic and surgical recurrence. In several models, targeting of MMP-9 attenuated chronic colitis, underlining the potential pathogenic role of this metalloproteinase in CD.[49,50] However, in CD several disease stages may be discerned and possibly, in the early acute phase, a small upregulation of MMP-9 in the macroscopically still normal tissue might be beneficial, whereas a large overexpression in the chronic active phase may lead to tissue ulceration and/or fibrosis. That would concur with our observation that the MMP and TIMP levels in the inflamed tissue are not prognostic to the diagnostic and surgical recurrence of CD. Recently, a protective role for MMP-2 (but not MMP-9) was demonstrated in experimental colitis, adding some credit to our hypothesis.[51] However, the exact mechanisms, proteolytic and nonproteolytic, by which the MMPs and TIMPs might be involved in the disease progression and recurrence is not completely clear. These factors are not only involved in tissue damage but also in intestinal wound healing, re-epitheliazation, myofibroblast and immune cell migration, scar formation, fibrogenesis, and neovascularization in the intestine, also after partial resection.[52-54] With regard to the clinical variables, we confirmed the effect of smoking also observed in previous publications.[15] Recently, cigarette smoke was shown to upregulate MMPs and induce a proinflammatory response.[55,56] We found, however, no relation between MMP and TIMP levels with smoking and the observed association of these proteins with recurrence was also independently significant in a multivariate model including smoking status. In summary, we have shown an association of the allele distribution at the TIMP-1 +372 T/C locus, the levels of TIMP-1, -2, and MMP-9 in non-inflamed tissue, and smoking habit with diagnostic and/or surgical recurrence of CD. Our results shed new light on the potential role of MMPs and TIMPs in the pathogenesis of recurrence/relapse in CD and may help in the identification of patients at risk, improving the effectiveness of current postoperative prophylactic treatment and disease management.[57,58]

Acknowledgments

The technical assistance of M. van den Berg, J.J. van der Reijden and W. van Duijn is greatly appreciated.

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Chapter 8

Summarizing Discussion

Introduction

The studies presented and discussed in this thesis focus on the clinical impact of Matrix Metalloproteinases (MMPs)-1, -2, -3, -9 and Tissue Inhibitor of Metalloproteinases (TIMP) in chronic inflammatory bowel disease (IBD) and evaluate the effect of increased TNF-α signaling on MMP and TIMP expression in IBD. First, expression and activity status of gelatinases MMP-2 and MMP-9 was determined in IBD compared to control intestinal mucosa. Second, the contribution of TNF-α in regulation of MMP-1, -2, -3, -9 and TIMP-1, -2 expression was assessed *ex vivo* and/or *in vitro*, and *in vivo* by administration of the chimeric TNF-α antibody infliximab®. Subsequently, we correlated the MMP and TIMP levels versus allelic composition at MMP, TIMP and TNF-α single nucleotide polymorphism (SNP) loci to determine the genetic influence on the respective proteins and indirectly on IBD susceptibility and CD phenotype. Finally, these SNPs and corresponding proteins in CD tissue were evaluated in a multivariate model, including selected clinical variables, for association with diagnostic and surgical recurrence. The results are summarized and discussed with reference to current literature.

Matrix metalloproteinases in Inflammatory Bowel Disease

A brief review concerning IBD and MMPs is provided in **chapter 1**. Two major forms of IBD are discerned, i.e., Crohn's disease (CD) and ulcerative colitis (UC). In CD inflammation is segmental and transmural and may involve any part of the gastro-intestinal tract with often the ileocecal area involved. In UC, the inflammation is continuous and confined to the mucosa. In most patients, disease is located in the left part of the colon, but pancolitis also frequently occurs. Annual incidence figures have risen since the second World War reaching the current plateau of 4-9 (CD) and 9-14 (UC) per 100,000, with concordant prevalence rates of 130-175 and 240-275 per 100,000 for CD and UC, respectively. Despite low prevalence, IBD is a debilitating disease with 10 years cumulative surgical resection rate between 25-60% and represents a high socio-economic burden. Therefore, much research is conducted aimed at identifying the etiology and

pathogenic mechanisms underlying IBD. Based on current evidence, impaired epithelial barrier function and insufficient innate immune response might result in overexposure to commensal bacteria, initiating and/or propagating the uncontrolled adaptive immune response observed in IBD. Disease is exacerbated by the presence of apoptosis resistant lymphocytes and neutrophils. The upregulation of a diverse array of pro-inflammatory cytokines and chemokines chronically activates mesenchymal, epithelial and immune cells and continuously attracts new leucocytes from the peripheral circulation. Tissue damage is mediated by reactive oxygen metabolites, perforin, granzymes, auto-antibodies, activated complement and action of serine proteases and MMPs. The MMPs constitute a group of neutral endoproteinases and belong to subclan M (also called metzincins) of the metalloprotease class and share sequential and structural motifs with ADAMs (A Disintegrin And Metalloproteinase) and ADAMTSs (A Disintegrin-like And Metalloproteinase with Thrombospondin type 1 motifS). Currently in humans, 23 different MMPs are recognized divided into collagenases, gelatinases, matrilysins, stromelysins, MT (membrane-type)-MMPs and a rest group. The MMPs have important functions in physiological and pathological matrix turnover and together are capable of cleaving a large array of matrix molecules including but not limited to collagen I-XI, proteoglycans, elastin, laminin, vitronectin, tenascin, entactin and fibronectin. Individual members are also capable of cleaving various non-structural molecules such as IL-8, Transforming Growth Factor-β (TGF-β) and insulin growth factor binding protein-3 (IGFBP-3). Pro-inflammatory cytokines, chemokines, growth factors and oxidants regulate the expression of MMPs in a wide array of cell types, dependent on various intracellular signaling pathways. Expression is also determined by DNA and histone modification of the MMP gene and allelic distribution at single nucleotide polymorphism (SNP) loci of the MMP promoter. In addition, translational and secretion rate are also tightly regulated. Most MMPs (except the MT-MMPs) are secreted as inactive zymogens and need to be activated by proteinases such as plasmin, thrombin, trypsin and/or (MT-)MMPs. Alternatively, substrate-induced conformational change may result in activation of the MMP, without loss of the pro-peptide. Active MMPs are inhibited in a 1:1 stoichiometric complex with a member of the Tissue Inhibitor of Metalloproteinases

(TIMP), a group of endogenous inhibitors with their expression tightly regulated, similar to MMPs, and besides MMP inhibition probably also involved in cell growth and/or apoptosis. MMPs may also be inhibited by a general anti-protease such as α2-macroglobulin and are removed from the circulation by lipoprotein related-receptor protein (LRP)-mediated uptake and degradation in lysosomal vesicles. Previous studies have shown an upregulation of several MMPs in inflamed IBD tissue and in animal models, inflammation score was significantly decreased by administration of MMP inhibitors, suggesting an important role for MMPs in IBD pathogenesis.

Expression and regulation of matrix metalloproteinases in Inflammatory Bowel Disease

In **chapter 2** a study is presented regarding the expression of gelatinase type MMP-2 and MMP-9 in inflamed and non-inflamed IBD. From 16 patients with CD, 14 patients with UC and 16 normal controls surgically resected mucosa was homogenized and used for ELISA measurements. Tissue protein levels of MMP-2 and MMP-9 were significantly increased in IBD dependent on inflammation status without major differences between CD and UC, corroborated by a respective increase in mRNA as demonstrated by RT-PCR. The active and pro-enzyme forms of both MMPs were increased, as determined by zymography, but in a bio-activity assay only MMP-9 activity was upregulated. Immunohistochemistry demonstrated MMP-2 expression primarily in the submucosa, whereas MMP-9 was associated with polymorphonuclear leucocytes. The results suggest a role for MMP-2 and MMP-9 in the stromal and inflammatory process, respectively, and confirm existing literature.[1-3]

In order to evaluate whether TNF-α-dependent regulatory mechanisms contribute to MMP-1, -2, -3, -9 and TIMP-1 and -2 expression in CD and UC an *ex vivo* model was used **(chapter 3)**. A protocol from Autrup *et al*. [4] was modified to culture mucosa isolated from surgically resected tissue specimens originating from adult human IBD and control patients. The 72 hrs cumulative expression of MMP-1, -2, -3 and -9 as determined by ELISA and/or immunocapture bio-activity

assay (BIA) in conditioned media was significantly increased in CD and UC compared to control intestinal tissue. Addition of infliximab, an anti-TNF-α antibody, decreased the production of MMP-1, -3 total and net activity, and of MMP–9 total antigen relative to TIMP, while PWM, a T/B cell activator, induced the expression of these MMPs, counteracted again by infliximab. Clearly, the expression of these MMP members in all examined tissues is partly mediated by TNF-α. Addition of PWM reduced the expression of MMP-2 by 50% while infliximab had no effect, illustrating a divergent regulatory pathway for this protein. Interestingly, the results for MMP-1, -3 and -9 appeared to be related to the allelic composition at selected SNP loci, for instance the effects of infliximab and PWM on MMP-9 production were much stronger in individuals carrying the CC compared to CT or TT genotype at SNP –1562. The results from these chapters point to a protective (MMP-2) and detrimental (MMP-1, -3 and -9) effect in tissue degeneration, as also suggested by others,[5] which is disturbed in CD *and* UC, and may be restored by infliximab. Subsequently, the contribution of TNF-α signaling to *in vivo* gelatinase expression in CD was determined (**chapter 4**). Serum MMP-2 and MMP-9 values were monitored in two CD patient cohorts with either fistulas or active disease (n = 38 and 31, respectively), each group formed by participants from an international trial and from an in-house study. Infliximab administration at 0, 2 and 6 weeks was paralleled by an increase of serum MMP-2 in CD patients with fistulas and at 10 weeks a subsequent decline was observed. Opposite to MMP-2, serum MMP-9 dropped during infliximab administration and gradually increased again after the last infusion, although not statistically significant and also observed in placebo treated groups. In the patients with active disease similar effects occurred, especially at higher infliximab dosage. Interestingly, immunohistochemical staining-intensity of MMP-9 positive polymorphonuclear leucocytes in intestinal mucosa dropped after infliximab infusion. Separate *in vitro* whole blood culture experiments demonstrated a lower MMP-2 level in blood obtained from CD compared to healthy volunteers. This level was not affected by LPS stimulation or infliximab treatment, presumably as no MMP-2 mRNA could be detected in blood leucocytes. However, production/secretion of MMP-9 was higher in CD leucocytes and LPS induced

MMP-9 expression in volunteers could be downregulated by infliximab at the mRNA but not protein level.

Association of MMPs and TIMPs with IBD susceptibility and phenotype

Chapter 5 describes the evaluation of total protein and activity levels of MMP-1, -2, -3, -9, TIMP-1 and TIMP-2 in a large group of tissue samples obtained from 122 CD, 20 UC and 62 control patients and also the association of these markers with CD phenotype. As determined by ELISA and/or BIA with/without prior *p*-amino-phenyl-mercuric-acetate (APMA) treatment, all MMPs were found to be upregulated in inflamed IBD tissue, especially MMP-3 (18.6 versus control value 0.0 U/mg total protein, $P<0.001$). Importantly, MMPs were also increased relative to TIMP paralleled by an increase in net MMP activity (for instance MMP-3: 5.7 vs 0 U/mg, $P<0.001$). Expression of MMP and TIMP was similar in inflamed CD with or without extensive fibrosis and in patients with development of fistulae during follow-up and was only slightly downregulated by treatment with immuno-suppressive corticosteroids and/or azathioprine.

This study was extended to include the analysis of MMP, TIMP and TNF-α genetic variability in relation with IBD susceptibility and CD phenotype, as reported in **chapter 6**. Genomic DNA was isolated from 134 CD and 111 UC patients versus 248 controls and analyzed by RFLP and tetra primer amplification refractory mutational system (ARMS) PCR. The T (in males) and TT (in females) allelic constitution at SNP TIMP-1 +372 T/C occurred more frequently in CD patients compared to controls. Levels of TIMP-1 protein in inflamed tissue were lower in male IBD but not CD patients carrying the T allele. No difference in TIMP-1 level was observed between women stratified to TIMP-1 genotype. The C allele at TIMP-1 SNP appeared associated with fistulae development during follow-up, but only in female CD patients. The 5T5T genotype at MMP-3 –1613 5T/6T was associated with reduced risk on colonic involvement at first presentation but increased risk on development of strictures during follow-up. The allelic composition at MMP-1, -2, -9, TIMP-2 and TNF-α SNPs was not related to CD or

UC susceptibility and phenotype, indicating a primary function as mediators/ effectors instead of initiators during IBD etiopathogenesis. As the control group partly consisted of colorectal carcinoma (CRC) patients donating macroscopically normal tissue at least 10 cm away from neoplasia, all analysis were repeated using only the healthy volunteers as controls, yielding similar results.

In the final study **(chapter 7)** previously determined allelic composition at selected MMP, TIMP and TNF-α SNPs and corresponding protein levels were related to clinical prognosis of CD. In a cohort of 87 CD patients, the T allele at TIMP-1 +372 T/C was found to be associated with a reduced median time lag between resection and re-resection (surgical recurrence), i.e., 10.7 versus 19.1 years, this effect was borderline non-significant in multivariate analysis using established clinico-pathological parameters. Low TIMP-1 levels in non-inflamed CD tissue (<2.88 ng/mg) were associated with increased risk on surgical recurrence, as were low levels of TIMP-2 and remarkably, MMP-9. No such effect was found for MMP and TIMP levels in inflamed tissue. Although positive smoking status was related to diagnostic and surgical recurrence, the results for MMP and TIMP are most likely not associated with smoking, since no correlation of these parameters with smoking status could be established. The results from chapter 6 and 7 point to TIMP-1 SNP + 372 T allele mediated downregulation of TIMP-1 protein levels shifting the balance between MMP and TIMP to a more proteolytic phenotype leading to enhanced CD susceptibility and worse CD prognosis.

MMP and TIMPs in IBD: disturbed balance?

As stated in the introduction of this thesis, IBD is considered an exaggerated immune response against commensal bacteria in genetically susceptible individuals. In this thesis evidence is provided of the pathophysiological importance of the presence of a disturbed balance of certain MMPs (e.g., MMP-2 and MMP-9) and TIMPs (Fig. 1, next page).

The chronic ulcers, fissures and fibrosis observed in IBD may reflect and result from an impaired mucosal barrier function. The basement membrane underlying epithelial and endothelial cells is of crucial importance in maintaining a stable tissue border and providing mechanical strength. It is primarily composed of multi-

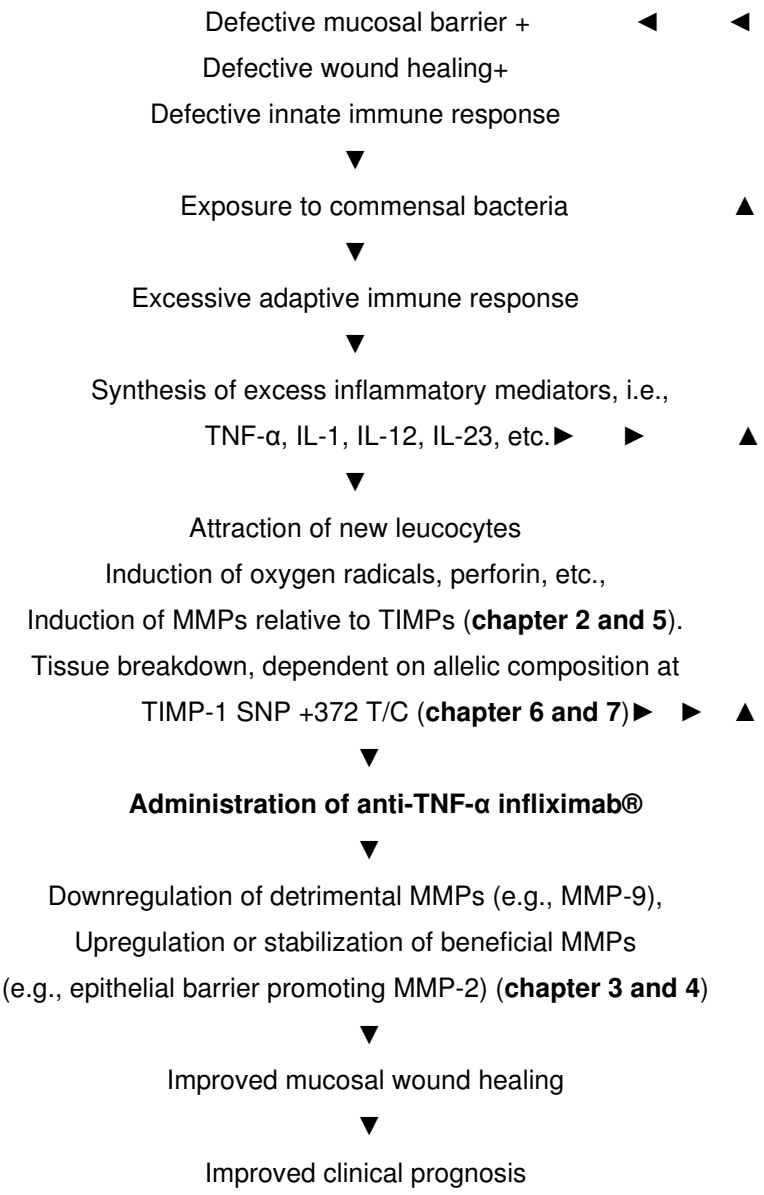

Figure 1. Schematic presentation of the contribution of MMPs and TIMPs in the pathogenesis of IBD. Application of anti TNF-α antibody infliximab® restores the pivotal MMP versus TIMP balance, promotes restoration of gut barrier function and improves clinical outcome of IBD patients.

layered collagen IV and laminin sheets, connected through nidogen and heparan sulphate proteoglycan perlecan, but may also contain other proteins such as anchoring collagen VII fibrils, fibulin, fibronectin, osteonectin, etc.[6] Basement membrane protein constituents are therefore potentially excellent candidates for involvement in IBD susceptibility and just recently an association of the "biological glue" extracellular matrix protein 1 with UC but not CD was found.[7] The persistent intestinal wounds seen in IBD might also reflect defective wound healing. After tissue injury, the fibroblast urokinase-type plasminogen activator receptor (uPAR) binds uPA, which cleaves blood-borne plasminogen into active plasmin. Plasmin not only cleaves fibrin, but also activates MMPs in close collaboration with other proteinases including MT-MMPs, chymotrypsin, etc., resulting in degradation of collagen lattices into a gelatin meshwork. Importantly, MMP activity is also tightly regulated by TIMPs, auto- and substrate-induced activation, inactivation and degradation.[8] Collagenolytic fragments are removed from the extracellular matrix (ECM) by uPAR-associated protein (uPARAP, also called endoprotein 180) mediated endocytosis and are degraded in lysosomal vesicles.[9-11] Epithelial cells and fibroblasts synthesize new collagen and other ECM constituents in close collaboration to reconstitute the matrix.[12,13] Also, the MMP-mediated breakdown of ECM provides epithelial cells with a mechanism to maintain their directionality during reepithelialization[14] and the cleavage of cell-cell and of cell-matrix interactions facilitates in cell migration.[15-19] In the process of remodeling, stored cytokines are released attracting leucocytes which clear the wound of pathogens. As stated in the introduction, MMPs are also involved in activating cytokines by proteolytic removal of inhibitory sequences. Finally, MMPs, like TIMPs, play a significant role in epithelial cell proliferation, differentiation and survival affecting the wound healing process as well. For instance, treatment of keratinocytes with MMP-2 silencing mRNA decreased proliferation and stimulated apoptosis, while silencing of MMP-9 exerted opposite effects, indicating different biological roles for these metalloproteinases depending on the cytokine micro-environment.[20-23] Several reports have indicated apparent overexpression of MMPs in non-healing chronic wounds.[24,25] Epidermal expression of MMP-1 in transgenic mice was associated with delayed wound healing.[26] Application of an hydro-active dressing

containing polyacrylate superabsorber particles removed MMPs from the wound bed and was associated with clinical improvement.[27] Perhaps the best illustration of what happens when excess metalloproteinases are present is provided by the snake venom metalloproteinases (SVMP). These proteins, closely related to the ADAMs (subclan M12) dissociate endothelial cells and dissolute the basement membrane, resulting in extensive hemorrhage.[28,29]

The process of fibrosis often seen in CD, may be the consequence of a too low MMP over TIMP ratio in combination with upregulated collagen production.[30-32] Alternatively, high levels of MMPs may facilitate fibroblast migration and also enhance fibrosis. Recently, MMP-3 was found to be involved in epithelial-mesenchymal cell transition, up-regulating collagen production and promoting the fibrotic phenotype.[33] The role of TIMP in wound healing is also multi-faceted. Inhibition of MMPs localizes and fine tunes remodeling of extracellular matrix. Production of TIMP-1 by fibroblasts promotes angiogenesis by preventing MMP-9 mediated release of anti-angiogenic tumstatin from collagen IV fibrils.[34] Besides the inhibition of MMPs, TIMP-2 is involved in the activation of MMP-2 and several TIMPs affect cell proliferation, differentiation and apoptosis.[35,36] Thus, wound healing is a subtle process requiring temporal and spatial coordination of collagen, MMP and TIMP synthesis. Importantly, both a low and a high MMP level at distinct time points may result in persistent non-healing ulcers. Besides the MMPs we studied, other MMPs may also be important, such as MMP-7 (matrilysin), MMP-8 (neutrophil collagenase) and MMP-12 (macrophage metallo-elastase). The ADAMs and ADAMTSs also play pivotal roles in matrix remodeling. For instance, ADAMTS knock-out mice experience less hepatic fibrosis when treated with carbon tetra-chloride and ADAM15-mediated T cell adhesion to epithelial cells enhances wound repair.[37,38] Our results show upregulation of selected MMPs relative to TIMP, resulting in increased net activity which can be partly restored by infliximab in CD *and* UC. Administration of this anti TNF-α drug to CD patients is associated with good clinical response and might be the consequence of downregulation of excess MMP-1, -3 and -9. From an MMP point of view, administration of infliximab to UC patients may also prove beneficial and indeed several studies in this patient group

have yielded promising results.[39] The concept of blocking MMP action in IBD patients by topical administration of selective synthetic MMP inhibitors is therefore appealing, but in light of their pleiotropic nature, as described above, should be considered with care. Selective MMP inhibition may result in functional substitution by other MMPs or ADAMs, considering their overlapping substrate specificity, while non-selective MMP inhibition may produce severe side-effects by inhibition of other MMPs, ADAMs and ADAMTSs beneficial to the healing process. Our studies on the allelic composition at the TIMP-1 SNP locus suggest T allele-mediated downregulation of TIMP-1 protein expression enhancing CD susceptibility and worsening CD prognosis. These results support the concept of MMP inhibition by topical administration of (recombinant) TIMP-1, but as MMP/TIMP levels were not related to clinical prognosis, the mechanism behind the C allele protective effect remains obscure and should be studied in more detail.

In summary, the studies described in this thesis provide new insights into the role of MMPs and TIMPs in the etiopathogenesis of CD and UC. The association of these proteins and the genetic variants with certain IBD phenotypes indicating an increased risk should be confirmed in prospective studies. Future work should also encompass the expression of other MMPs, ADAM (TS)s and TIMPs in intestinal tissue, the distribution of SNPs in corresponding genes and focus on the identification of MMP degradation fragments, physiological activation cascade, inhibitors and substrate specificity to obtain a detailed mechanistic view of metalloproteinase action in IBD. Ultimately, these studies might result in the development of novel therapeutic drugs in CD and UC, and perhaps other pathologies as well, based on the inhibition or instead activation of one or more members of the versatile metalloproteinase family.

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Chapter 9

Samenvattende discussie

Inleiding

In de periode 1999-2004 is door de onderzoeksgroep waarin ik participeerde een aantal studies verricht naar de invloed van matrix metalloproteïnasen (MMP) en weefsel inhibitoren van metalloproteïnasen (TIMP) op het verloop van inflammatoir darmlijden (*Inflammatory Bowel Disease*, IBD). Tevens is hierbij de rol onderzocht van verhoogde concentraties aan het ontstekingsbevorderende cytokine Tumor Necrosis Factor-alpha (TNF-α). Al dit onderzoek heeft geresulteerd in de totstandkoming van het proefschrift dat voor u ligt. Eerst is gekeken naar de aanmaak en activiteit van de gelatinasen MMP-2 en MMP-9 in IBD versus macroscopisch normaal darmslijmvlies (*mucosa*). Vervolgens hebben we *ex vivo, in vivo* en/of *in vitro* de regulatie van MMP-1, -2, -3 en -9, alsmede TIMP-1 en -2, door TNF-α bestudeerd, hierbij gebruik makend van het TNF-α neutraliserende antilichaam infliximab. Van een groot aantal IBD patiënten is de genetische compositie bepaald op vaststaande plekken (*loci*) in het MMP en TIMP DNA (analyse van single nucleotide polymorphisms, SNPs) waarna een associatie werd gelegd met de corresponderende eiwit-aanmaak en met IBD susceptibiliteit (*gevoeligheid*), alsmede de uiterlijke verschijningsvorm (*fenotype*) van de ziekte van Crohn (CD). Tenslotte werd in een multivariabele statistische analyse, temidden van een aantal geselecteerde klinische parameters, de SNP compositie en de expressie van MMP- en TIMP-eiwitten gerelateerd aan de snelheid van het optreden van een diagnostisch- en chirurgisch-recidief in patiënten met de ziekte van Crohn.

Matrix metalloproteïnasen in IBD

In **hoofdstuk 1** wordt een algemene beschouwing gegeven over IBD en MMPs. Binnen IBD worden twee vormen onderscheiden, te weten colitis ulcerosa (*ulcerative colitis*, UC) en de ziekte van Crohn (*Crohn's disease*, CD). In patiënten met CD worden zieke stukjes darm afgewisseld met gezonde stukken en de ontsteking kan zich dwars door de hele darmwand manifesteren (*transmuraal*). In principe kan elk deel van het maag-darmkanaal worden aangetast ("van mond tot kont"), in de praktijk blijkt met name de overgang van de dunne in de dikke darm

(*ileocaecaal* gebied) extra gevoelig. Bij patiënten met UC is alleen de dikke darm (het colon) aangetast, het ziekteproces is hier meer continu en beperkt zich tot het slijmvlies zonder diepere lagen van de darm aan te tasten. Na de Tweede Wereldoorlog is het jaarlijkse aantal nieuw gediagnosticeerde patiënten (*incidentie*) toegenomen en bedraagt nu ongeveer 4-9 per 100.000 inwoners in het geval van CD en 9-14 voor UC. Daardoor is ook het aantal mensen dat op enig moment één van beide ziektes heeft (ofwel: de *prevalentie*) toegenomen tot 130-175 voor CD en 240-275 voor UC per 100.000 inwoners. Een willekeurige IBD patiënt doet meestal een groot beroep op de medische zorg in termen van medicijngebruik, ziekenhuisopname en operaties (25-60% moet geopereerd worden binnen 10 jaar na diagnose). Er is dan ook veel geïnvesteerd om de oorzaken te achterhalen van IBD en ook om betere behandelmethoden te vinden. De huidige hypothese luidt dat IBD wordt veroorzaakt door een verminderde epitheliale-functie in combinatie met een verminderde niet-specifieke immuunfunctie. Dit resulteert in te hoge concentraties bacteriën in de darmwand, waardoor ons adaptieve -specifieke-immuunsysteem in de darm continu wordt gestimuleerd, met alle gevolgen van dien. Het ziekteproces kan hierbij nog verergerd worden omdat is aangetoond dat bepaalde immuuncellen in patiënten met IBD, bijvoorbeeld lymfocyten en neutrofielen, veel minder gevoelig zijn geworden voor geprogrammeerde celdood (*apoptose*) en aldus langer hun vernietigende werk kunnen blijven doen. De geactiveerde immuuncellen scheiden zeer veel cytokines en chemokines af, die andere cellen zoals epitheel en fibroblasten eveneens activeren en ervoor zorgen dat nieuwe immuuncellen vanuit het bloed in de darmwand terecht komen. De bacteriën worden bestreden met reactieve zuurstofradicalen, perforine en granzymes (bepaalde celdodende eiwitten), antilichamen, complement en proteasen, zoals de serine-proteasen en MMPs. Deze stoffen zijn echter in grote hoeveelheden niet alleen schadelijk voor bacteriën, maar zijn dat ook voor het eigen darmweefsel. Hierdoor wordt de darmwand nog verder verzwakt, komen nog meer bacteriën binnen waardoor een spiraal naar beneden ontstaat die vaak alleen nog maar kan worden onderbroken door het chirurgisch verwijderen van het zieke stuk darmweefsel.

Matrix Metalloproteïnasen (MMPs) knippen andere eiwitten kapot (protease functie) en doen dat het best bij een pH van rond de 7. In mensen zijn tot nu 23 verschillende MMPs gevonden, welke worden onderverdeeld in zogenaamde collagenasen, gelatinasen, matrilysinen, stromelysinen, membraan-gebonden MMPs en een overige groep. In elk gezond persoon spelen MMPs een belangrijke rol in processen waarbij weefsel moet worden vervangen en/of opnieuw gemodelleerd, zoals bij de groei van een foetus, de continue vervanging van de darmwand en opperhuid, wondheling, etc. Tevens spelen ze een belangrijke rol in ziektes, zoals bij diverse vormen van kanker, waar ze de infiltratie en uitzaaiing van tumorcellen kunnen versterken of juist tegengaan. Elk MMP heeft zijn eigen voorkeur voor welke eiwitten hij het beste knipt (*substraatspecificiteit*). Gezamenlijk zijn de MMPs in staat een groot aantal eiwitten te knippen, zoals collageen I-XI, proteoglycanen, elastine, laminine, vitronectine, tenascine, entactine and fibronectine. Naast bovengenoemde structuur-eiwitten kunnen bijvoorbeeld ook cytokines als Interleukine 8 (IL-8) en Transforming Growth Factor-beta (TGF-β) geknipt worden. De door de MMPs bewerkstelligde knip in een eiwit is vaak een eerste stap in de afbraak hiervan, maar kan juist ook een eiwit activeren, bijvoorbeeld in het geval van IL-8. Gezien hun potentieel gevaarlijke rol wordt de aanmaak van MMPs in diverse cellen op verschillende niveaus strikt gereguleerd. Cytokines, chemokines, groeifactoren en oxidatie-status kunnen de aanmaak van MMPs omhoog of juist naar beneden schroeven, vaak in interactie met elkaar en ook nog eens afhankelijk van celtype. Methylering en acetylering van DNA en van DNA-bindende histon-eiwitten spelen ook een belangrijke rol, evenals de genetische compositie op SNP loci in het DNA gebied met de on/off switch voor MMP-productie (*promoter gebied*). Verder is het MMP messenger-RNA (mRNA) vertaalproces streng gereguleerd evenals de uitscheiding (*excretie*) van MMPs door cellen. De meeste MMPs worden bovendien geproduceerd in een inactieve vorm en moeten geactiveerd worden door andere proteasen als plasmine, thrombine, trypsine of bijvoorbeeld andere MMPs, uiteraard weer op een gecoördineerde en gecontroleerde manier. Geactiveerde MMPs zijn nooit lang actief, ze binden namelijk al snel aan een lid van de TIMP groep en worden hierdoor geremd. Op dit moment zijn in mensen vier verschillende TIMPs

gevonden. De aanmaak van deze eiwitten en de controle hierop lijkt wel wat op dat van de MMPs, al zijn er individuele verschillen. Naast MMP remming zijn TIMPs ook betrokken bij processen als celgroei, -proliferatie en -dood. Andere, meer algemene niet-MMP-specifieke proteasen kunnen ook binden aan MMPs en deze remmen. Het lipoproteïne gerelateerde-receptor proteïne (LRP) verwijdert tenslotte MMPs uit de circulatie waarna deze intracellulair worden afgebroken in zogenaamde lysosomale blaasjes. Voorgaande studies hebben een grote toename laten zien in de aanmaak van één of meer MMP-leden in de darmwand van IBD patiënten. In muizen- en ratten-IBD-modellen blijkt tevens dat de ontstekingsscore naar beneden kan worden gebracht door de toediening van synthetische MMP-remmers, hetgeen een grote rol voor MMPs in het ziekteproces van IBD suggereert.

Aanmaak en regulering van matrix metalloproteïnasen in IBD

In de eerste studie (**hoofdstuk 2)** van dit proefschrift is gekeken naar de expressie van gelatinase-type MMP-2 en MMP-9 in IBD weefsel. Van 16 patiënten met CD, 14 met UC en 16 normale controles werd operatief verkregen darmmucosa gehomogeniseerd en met behulp van de ELISA techniek geanalyseerd op het voorkomen van beide gelatinasen. Zowel MMP-2 als MMP-9 eiwit zijn, afhankelijk van de ontstekingsgraad, verhoogd aanwezig in IBD weefsel, en met behulp van de RT-PCR techniek kon dit patroon ook op mRNA nivo worden aangetoond. Zymografie analyse liet zien dat de actieve- en pro-enzymvormen van beide MMPs verhoogd aanwezig zijn, maar in een bio-activiteitsassay was alleen MMP-9 significant verhoogd. Met behulp van immunohistochemie kon worden aangetoond dat MMP-2 voornamelijk in het bindweefsel tussen mucosa en darmspier (*submucosa*) wordt aangemaakt, terwijl de bron van MMP-9 productie de polymorfonucleaire leukocyten (PMNL) lijken te zijn. Deze resultaten zijn in overeenstemming met eerder gepubliceerde studies en duiden op een rol van MMP-2 en MMP-9 in, respectievelijk, het stromale- en inflammatoire-proces.

In een vervolgstudie werd onderzocht of er een TNF-α-afhankelijkheid in de regulatie van MMP-1, -2, -3 en -9 en TIMP-1 en -2 bestaat (**hoofdstuk 3)**. Direct na operatie werd restant darmmucosa van volwassen IBD- en controle-patiënten

opgeknipt en in kleine stukjes gedurende drie dagen gekweekt onder gecontroleerde omstandigheden. De 3-daags cumulatieve productie van alle onderzochte MMPs in het kweek medium, bepaald door middel van ELISA en/of BIA, was significant groter in IBD-vergeleken met controleweefsel. Het toevoegen van het TNF-α-neutraliserende antilichaam infliximab resulteerde in een afname van MMP-1 en -3 totale- en netto activiteit, terwijl ook de expressie van MMP-9 relatief ten opzichte van TIMP een afname liet zien. Toevoeging van Pokeweed Mitogen (PWM), een B/T lymfocyt activator, induceerde de expressie van de genoemde MMPs, welke voor een deel werd tegengegaan door gelijktijdige aanwezigheid van infliximab. Het lijkt er dus op dat in geval van MMP-1, -3 en -9 de productie gedeeltelijk wordt bepaald door TNF-α, zowel in IBD- als controleweefsel. Echter, toevoeging van PWM reduceerde de MMP-2 productie met 50%, terwijl infliximab geen enkel effect had, hetgeen erop wijst dat in geval van dit gelatinase andere regulatie-mechanismen een grotere rol spelen. De regulatie door TNF-α van MMP-1, -3 en -9 lijkt bovendien afhankelijk te zijn van de genetische compositie op SNP plekken. De effecten van infliximab en PWM op MMP-9 productie waren bijvoorbeeld veel groter in patiënten met het CC vergeleken met het CT of TT genotype op SNP MMP-9 –1562 C/T. Deze resultaten wijzen op een beschermende rol van MMP-2 en een beschadigende rol van MMP-1, -3 en -9 in weefselmodellering, welke balans verstoord is in CD en UC en hersteld zou kunnen worden door infliximab.

Vervolgens hebben wij in de patiënt zelf (*in vivo*) gekeken naar het aandeel van TNF-α in de regulatie van gelatinase-type MMP-2 en MMP-9, zoals beschreven in **hoofdstuk 4**. De serumwaardes van deze eiwitten werden gemeten in twee CD patiëntengroepen. De ene groep werd behandeld voor fistels (buisvormige zweren tussen darmen onderling of tussen darm en huid) en de andere groep werd behandeld voor ontstekingen in de darm (*n* =38 en 31, respectievelijk). De patiënten met fistels kregen infliximab toegediend op t = 0, 2 en 6 weken. Gedurende deze periode nam het serum MMP-2 geleidelijk toe, om daarna weer te dalen. In tegenstelling tot MMP-2 nam het serum MMP-9 juist af gedurende de eerste 6 weken, om daarna weer toe te nemen. In de patiëntengroep met actieve ziekte werden dezelfde effecten geregistreerd, vooral bij hogere infliximab doses.

In deze groep bleek ook de intensiteit van immunohistochemische aankleuring van MMP-9 positieve PMNLs te verminderen na infliximab infusie. In aparte bloedkweek-experimenten bleek de hoeveelheid van MMP-2 in CD lager te liggen vergeleken met dat van controles. Deze spiegels konden niet worden beïnvloed door toevoeging van het immuno-activerende lipopolysaccharide (LPS) of infliximab, waarschijnlijk vanwege het ontbreken van MMP-2 mRNA in de bloedleukocyten. De productie van MMP-9 lag echter hoger in het bloed van CD-patiënten versus dat van controles, kon worden gestimuleerd met LPS en, althans op het mRNA nivo, weer naar beneden worden gebracht met infliximab.

Associatie van MMPs en TIMPs met IBD susceptibiliteit en fenotype

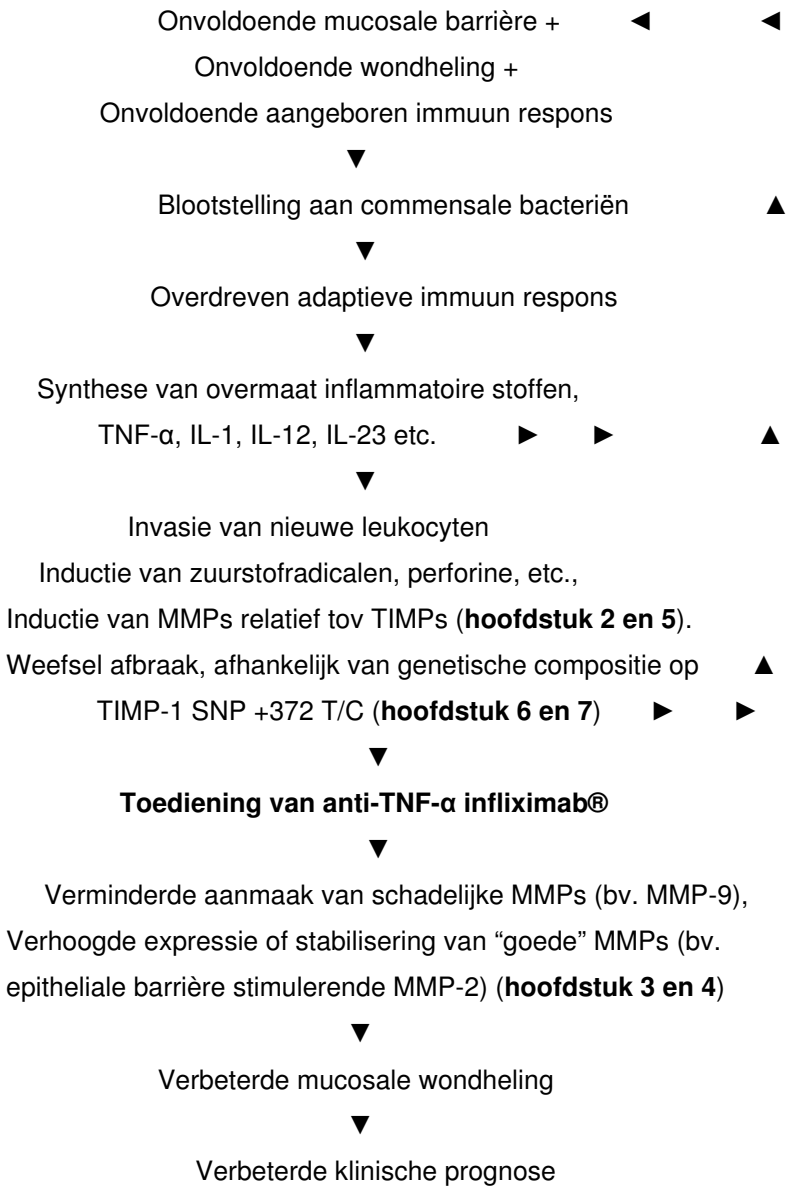
Gezien de veranderingen die waren gevonden in de expressie en regulatie van de MMPs en TIMPs in IBD was het van belang te onderzoeken of deze werden gestuurd door het fenotype van de ziekte en de genetische opmaak van de IBD-patiënten. Daarom werd eerst een studie uitgevoerd als beschreven in **hoofdstuk 5,** waarin de totale hoeveelheid eiwit en activiteit van MMP-1, -2, -3 en, -9 alsmede TIMP-1 en -2 is gemeten in darmmucosa van 122 CD, 20 UC en 62 controle patiënten in relatie tot ziekte actviteit en bij CD-patiënten in relatie tot het fenotype. Alle MMPs bleken verhoogd aanwezig te zijn in ontstoken IBD-weefsel, aangetoond met ELISA en/of BIA met voorafgaande p-amino-phenyl-mercuric-acetate (APMA) activering. De MMPs waren ook verhoogd relatief vergeleken met TIMPs, wat gepaard ging met een verhoging van de netto MMP-activiteit (aangetoond door middel van BIA zonder voorafgaande APMA behandeling). Zo was bijvoorbeeld de totale MMP-3 hoeveelheid 18,6 Units (U)/mg en de netto MMP-3 activiteit 5,7 U/mg in ontstoken IBD-weefsel, terwijl in controle-weefsel MMP-3 niet eens gemeten kon worden (0,0 U/mg, P<0,001). In ontstoken CD-weefsel was de mate van bindweefselvorming (*fibrosering*) niet van belang voor de expressie van MMPs en TIMPs. Evenmin was de aanmaak van deze eiwitten niet geassocieerd met het wel of niet optreden van fistels gedurende de follow-up. Tenslotte bleek de expressie van alle MMPs en TIMPs slechts gedeeltelijk naar

beneden gebracht te kunnen worden door middel van behandeling van de patiënt met immunosuppresieve medicatie als corticosteroïden of azathioprine. Vervolgens hebben we gekeken of wellicht genetische variabiliteit in MMP, TIMP of TNF-α een invloed zou kunnen hebben op eiwit-expressie en op IBD susceptibiliteit en CD fenotype **(hoofdstuk 6)**. Genomisch DNA werd gewonnen uit het bloed en/of operatie weefsel van 134 CD en 111 UC patiënten versus 248 controles, waarna het werd geanalyseerd met behulp van de restriction fragment length polymorphism (RFLP)-techniek en met tetra primer amplification refractory mutational system (ARMS)-PCR. Het T-(mannen) en TT-(vrouwen)genotype op SNP TIMP-1 +372 T/C bleek vaker voor te komen in CD patiënten vergeleken met controles. De TIMP-1 eiwit expressie was lager in het ontstoken weefsel van mannelijke IBD patiënten met het TIMP-1 T allel vergeleken met patiënten die drager zijn van het C allel, maar dit kon voor CD patiënten niet worden aangetoond en evenmin voor vrouwen. Het TIMP-1 C allel lijkt geassocieerd met de (latere) ontwikkeling van fistels in vrouwelijke CD patiënten. Het 5T5T genotype van SNP MMP-3 –1613 5T/6T is geassocieerd met een lagere kans op aanwezigheid van het CD ziekteproces in het colon tijdens de eerste diagnose, maar ook met een verhoogde kans op de ontwikkeling van stricturen gedurende follow-up. De genetische variabiliteit op geselecteerde SNP loci van MMP-1, -2, -9, TIMP-2 en TNF-α was niet geassocieerd met CD of UC susceptibiliteit of fenotype, hetgeen een primaire rol voor deze eiwitten verderop in het IBD ziekteproces in plaats van aan het begin ervan veronderstelt. De controle-groep bestond voor een deel uit colorectale carcinoompatiënten, waarvan uit het operatiepreparaat wat normaal darmmucosa op ongeveer 10 cm van het kankergezwel was genomen. Aangezien dit van invloed had kunnen zijn op de genetische resultaten werden alle analyses herhaald met alleen de gezonde vrijwilligers in de controle-groep, wat dezelfde uitkomsten gaf. In de afsluitende studie **(hoofdstuk 7)** zijn de resultaten van de genetische variabiliteit op bovengenoemde MMP, TIMP en TNF-SNPs alsmede eiwitniveaus gekoppeld aan de klinische prognose van CD. Patiënten met het T-allel op SNP TIMP-1 +372 T/C bleken na operatie sneller (10,7 jaren) wederom onder het mes te moeten vergeleken met patiënten zonder deze SNP variant (19,1 jaren). Het effect verloor maar net zijn significantie in een multivariate analyse

gebruik makend van geselecteerde klinisch-pathologische kenmerken als geslacht, leeftijd bij operatie, etc. Verder bleek een lage TIMP-1 expressie (<2,88 ng/mg) in niet-ontstoken CD-weefsel geassocieerd met een verhoogde kans op operatieve resectie. Dit werd tevens gevonden voor TIMP-2, en merkwaardig genoeg, ook voor MMP-9. Het MMP of TIMP nivo in ontstoken weefsel was in dit opzicht niet van belang. In onze analyses bleek het roken van sigaretten een belangrijke prognostische variabele voor een diagnostisch- en chirurgisch- recidief. Onze MMP en TIMP resultaten hebben echter waarschijnlijk niets te maken met het roken, omdat geen enkele relatie tussen MMPs, TIMPs en rookgedrag kon worden aangetoond, ook niet in de multivariate analyses. Een belangrijke conclusie die getrokken kan worden uit hoofdstukken 6 en 7 is dat het TIMP-1 SNP +372 T allel mogelijk de expressie van TIMP-1 negatief beïnvloedt, waardoor de balans tussen MMP en TIMP verschuift naar een meer proteolytisch fenotype wat de susceptibiliteit voor CD verhoogt en tevens leidt tot een slechtere prognose van CD.

MMPs en TIMPs in IBD: een verstoord evenwicht?

De huidige hypothese luidt dat IBD veroorzaakt wordt door een overdreven immuunreactie tegen commensale bacteriën in genetisch vatbare personen. In dit proefschrift is aangetoond dat een verstoord evenwicht tussen MMPs en TIMPs betrokken is in de pathogenese van IBD, zie figuur 1, volgende bladzijde. De chronische zweren, fistels, insnoeringen (*fissuren*) en fibrosering, zoals die kunnen voorkomen in de darm van IBD patiënten, kunnen het gevolg zijn van een verminderde mucosale barrière. De zogenaamde basaalmembraan onder epitheel- en endotheelcellen verleent steun, flexibele mechanische weerstand en scheidt weefselcompartimenten. Het bestaat uit meerdere lagen collageen IV en laminine, met elkaar verbonden door nidogeen en heparansulfaat-proteoglycaan perlecan, maar bevat ook andere eiwitten als collageen VII fibrillen, fibuline, fibronectine, osteonectine, enz. Basaalmembraan-eiwitten zouden heel goed betrokken kunnen zijn bij IBD susceptibiliteit en in lijn hiermee is recent een genetische associatie van extracellulaire matrixproteïne-1 (een biologische “lijm”) met UC maar niet met CD gevonden.



Figuur 1. Schematische presentatie van MMPs en TIMPs in de pathogenese van IBD. Toediening van het anti TNF-α antilichaam infliximab® herstelt de cruciale MMP versus TIMP balans, stimuleert herstel van de darm barrière functie en verbetert de klinische prognose van IBD patiënten.

De chronische wonden in IBD patiënten zouden ook het gevolg kunnen zijn van niet-adequate wondheling. Verwonding resulteert in eerste instantie in de snelle vorming van het welbekende bloedkorstje, waarna het langzamere weefselherstel kan beginnen. Als eerste stap hiertoe bindt de urokinase-type plasminogen activator-receptor (uPAR) het uPA, welke vervolgens plasminogeen knipt tot actief plasmine. Dit laatste eiwit knipt niet alleen het fibrine (wat het tijdelijk bloed korstje vormt) maar activeert, in nauwe samenwerking met andere proteïnasen als membraan-gebonden MMPs en chymotrypsine, ook verschillende leden van de MMP familie waardoor beschadigd collageen matrix afgebroken kan worden tot een gelatine “soep”. Dit proces verloopt normaal gesproken onder strenge controle: de MMP activiteit wordt gereguleerd door TIMPs, auto- en substraat geïnduceerde-activering, inactivering en degradatie. De collageen fragmenten worden uit de extracellulaire matrix verwijderd door uPAR-geassocieerde-proteïne (uPARAP) gemedieerde endocytose en worden vervolgens vernietigd in lysosomale blaasjes. Tegelijkertijd maken epitheliale cellen en fibroblasten nieuw collageen en andere matrixcomponenten, wat uiteindelijk mede het nieuwe weefsel vormt en het bloedkorstje vervangt. De MMPs spelen niet alleen een belangrijke en direkte rol in weefsel-hermodellering, maar zijn ook van belang in het faciliteren van epitheliale proliferatie, differentiatie, apoptose en daarmee in het proces van epitheliale wondsluiting. Hierbij kunnen diverse MMPs een verschillende rol spelen. Zo stimuleert MMP-2 epiheliale cellen en helpt ze overleven, terwijl voor MMP-9 juist een tegenovergestelde rol is weggelegd. Verder is het zo dat door het hermodelleren van weefsel de in matrix opgeslagen cytokines vrijkomen, die na activering door MMPs nieuwe leukocyten naar de wondplek lokken. Verschillende studies hebben aangetoond dat chronische niet-helende wonden een overmaat aan MMPs hebben. Kunstmatige overexpressie van MMP-1 in transgene muizen was geassocieerd met een vertraagde wondheling. Applicatie van een hydroactieve dressing met superabsorberende polyacrylaat-deeltjes verwijderde MMPs uit de wond en was geassocieerd met een verbeterde genezing. Wellicht de beste illustratie van wat er kan gebeuren in geval van een overmaat aan MMPs wordt gegeven door de snake venom (*slangengif*) metalloproteïnasen. Deze

eiwitten, aanwezig in slangengif, knippen endotheelcellen van mekaar en lossen de basaalmembraan op, met ernstige bloeduitstortingen tot gevolg.

Fibrose, vaak gezien in CD, zou het gevolg kunnen zijn van te weinig MMP in verhouding tot TIMP, in combinatie met een verhoogde collageenproductie. Een alternatieve verklaring zou kunnen zijn dat hoge niveaus van MMPs de migratie van fibroblasten zouden kunnen faciliteren en op die manier fibrose juist zouden kunnen stimuleren. Recent heeft een groep auteurs aangetoond dat MMP-3 epitheliale cellen in cellen met mesenchymale eigenschappen kan transformeren, waardoor hun collageenproductie werd verhoogd en het fibrotische fenotype werd gestimuleerd. De rol van TIMPs kent eveneens vele gezichten. Het remmen van MMPs is belangrijk voor het fine tunen van de weefselmodellering. Aan de andere kant is TIMP-2 noodzakelijk voor de activatie van MMP-2 en van verscheidene TIMPs is aangetoond dat ze een rol spelen bij celdifferentiatie, proliferatie en apoptose. Het moge duidelijk zijn dat wondheling een subtiel proces is, waarbij een gecoördineerde aanmaak van MMPs, TIMPs en collageen een belangrijk vereiste is: zowel te weinig als teveel MMPs op verschillende tijdstippen kan resulteren in chronische zweren. Andere MMPs, zoals MMP-7 (matrilysine), MMP-8 (neutrofiel collagenase) en MMP-12 (macrofaag metallo-elastase) spelen mogelijk ook een belangrijke rol, evenals aan MMP-gerelateerde eiwitten als ADAMs (A Disintegrin And Metalloproteïnase) en ADAMTSs (A Disintegrin-like And Metalloproteïnase with Thrombospondin type 1 motifS).
Onze resultaten wijzen op een overmaat aan MMP productie ten opzichte van TIMPs, resulterend in een verhoogde netto-activiteit en beschadiging van het darmweefsel, wat kan worden tegen gegaan door toediening van infliximab in zowel CD als UC patiënten. De goede klinische respons die patiënten geven na infliximab behandeling kan het gevolg zijn van een verminderde aanmaak van MMP-1, -3 en -9. Van hieruit bezien zou behandeling van UC patiënten met infliximab ook een goede optie zijn, en de resultaten van verschillende pilot-studies wijzen hier ook op. Het is verleidelijk om synthetische MMP-remmers aan IBD patiënten te geven, maar gezien de veelzijdigheid van deze eiwitten is voorzichtigheid op zijn plaats. Selectieve MMP-remming zou kunnen resulteren in

functionele substitutie door andere MMPs of ADAM(TS)s, gezien hun overlappende substraatspecificiteit. Niet-specifieke remming zou daarentegen kunnen leiden tot ernstige bijwerkingen door remming van MMPs en ADAM(TS)s welke juist bevorderlijk zijn voor het genezingsproces, zoals MMP-2. Onze resultaten voor wat betreft de genetische compositie op het TIMP-1 SNP +372 T/C suggereren een T allel veroorzaakte daling van TIMP-1 eiwitproductie, zodat MMPs meer vrij spel krijgen, waardoor de susceptibiliteit voor CD wordt verhoogd en de prognose negatief wordt beïnvloed. Remming van de overmaat MMPs met recombinant TIMP is daarom wellicht een interessant alternatief. Echter, omdat de MMP/TIMP ratios niet gerelateerd zijn aan de klinische prognose, is het niet duidelijk waar het positieve effect van het C allel vandaan komt, dit zal dus eerst bestudeerd moeten worden.

Samenvattend: het in dit proefschrift beschreven onderzoek geeft nieuwe inzichten in de rol van MMPs en TIMPs in de etiopathogenese van IBD. Toekomstige studies moeten onderzoek omvatten naar de expressie van andere MMPs, ADAM(TS)s en TIMPs in darmweefsel, alsmede de genetische compositie op corresponderende relevante SNPs. Daarnaast moet er een focus gelegd worden op de identificatie van MMP degradatie-fragmenten, de fysiologische activeringscascade, remmers en substraatspecificiteit om een gedetailleerd mechanistisch inzicht te verkrijgen in metalloproteïnase-activiteit in IBD. Uiteindelijk kunnen deze studies resulteren in het ontwikkelen van nieuwe medicijnen voor CD en UC, en wellicht andere immunopathologiën, gebaseerd op de remming dan wel activering van één of meer leden van de veelzijdige matrix metalloproteïnasen familie.

Nawoord

Dit proefschrift is tot stand gekomen met de hulp van velen. Ik wil op deze plaats als eerste de wetenschappelijk medewerkers van het laboratorium Maag-, Darm- en Leverziekten (Hein, Izäk, Qiang, Wim, Marij, Annie, Jan-Paul (†), Kees, Marlies, Johan en Eveline) bedanken voor hun steun en inzet. Daarnaast ben ik de afdeling Pathologie zeer erkentelijk voor het beschikbaar stellen van resectieweefsel, TNO (Roeland en Jan) voor het faciliteren van de MMP ELISAs en BIAs, het personeel van het medisch archief voor hun assistentie bij het doorzoeken van de talrijke medische dossiers en de artsen in opleiding (Tomas, Eduard) voor de stimulerende discussies. Tenslotte gaat mijn dank uit naar mijn familie (pap, mam en Helma), schoonfamilie en vrienden voor hun morele en motiverende steun gedurende het hele traject en natuurlijk naar mijn partner Pascale niet alleen voor haar rol als steun en toeverlaat maar ook voor het vele redactionele werk en het ontwerp van de kaft.

Curriculum vitae

De auteur van dit proefschrift werd op 29 september 1974 geboren in Amsterdam. In 1993 werd het VWO diploma behaald aan het Augustinus College in Groningen waarna aansluitend werd gestart met de opleiding biologie aan de Landbouw Universiteit Wageningen. Gedurende het afstudeervak onder begeleiding van ing. A.H. Westphal en Dr. A. de Kok werd onderzoek gedaan aan het *Neisseria meningitides* lipoamidedehydrogenase, onderdeel van het pyruvaatdehydrogenase-complex van deze bacterie. Tijdens de afsluitende stage in het St Radboud Ziekenhuis te Nijmegen onder begeleiding van Dr. M.R. Bernsen en Dr. G.N. van Muijen is onderzoek verricht ten behoeve van de moleculaire karakterisatie van melanoom infiltrerende T-lymfocyten. Na het behalen van het doctoraal diploma in 1998 met afstudeerrichting celbiologie werd in 1999 op de afdeling Maag-, Darm- en Leverziekten van het Leids Universitair Medisch Centrum aangevangen met het door de Maag Lever Darm Stichting gesubsidieerde promotie-onderzoek naar de rol van matrix metalloproteinases bij chronisch inflammatoir darmlijden onder begeleiding van Dr. ir. H.W. Verspaget en Prof. dr. C.B.H.W. Lamers, hetgeen heeft geresulteerd in een aantal publicaties en de totstandkoming van dit proefschrift. Tegenwoordig is de auteur als junior medewerker in dienst bij Domenica en verricht pensioen-specialistische werkzaamheden voor diverse verzekeraars.