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NOVEL MATRIX METALLOPROTEINASES IN INTESTINAL INFLAMMATION AND IN CANCER OF THE GASTROINTESTINAL TRACT

Ville Bister

Academic dissertation

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To my grandmother

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List of original publications

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-V. In addition some unpublished material is included.

I. Bister VO, Salmela MT, Karjalainen-Lindsberg ML, Uria J, Lohi J, Puolakkainen P, Lopez-Otin C, Saarialho-Kere U. Differential expression of three matrix metalloproteinases, MMP-19, MMP-26, and MMP-28, in normal and inflamed intestine and colon cancer. *Dig Dis Sci* 49:653-61, 2004

II. Bister V, Salmela MT, Heikkila P, Anttila A, Rintala R, Isaka K, Andersson S, Saarialho-Kere U. Matrilysins-1 and -2 (MMP-7 and -26) and metalloelastase (MMP-12), unlike MMP-19, are up-regulated in necrotizing enterocolitis. *J Pediatr Gastroenterol Nutr* 40:60-6, 2005

III. Bister V, Kolho KL, Karikoski R, Westerholm-Ormio M, Savilahti E, Saarialho-Kere U. Metalloelastase (MMP-12) is upregulated in the gut of pediatric patients with potential celiac disease and in type 1 diabetes. *Scand J Gastroenterol* 40:1413-22, 2005

IV. Bister V, Mäkitalo L, Jeskanen L, Saarialho-Kere U: Expression of MMPs-9, -10, and TNFα, and lack of epithelial MMP-1 and -26, characterize pyoderma gangrenosum. *J Cutan Pathol,* in press

V. Bister V, Skoog T, Virolainen S, Kiviluoto T, Puolakkainen P, Saarialho-Kere U. Increased expression of matrix metalloproteinases-21 and -26, and TIMP-4 in pancreatic adenocarcinoma. Submitted

Abbreviations

BM	basement membrane
CD	celiac disease
cDNA	complementary deoxyribonucleic acid
CrD	Crohn´s disease
ECM	extracellular matrix
EGF	epidermal growth factor
EMA	endomysium antigen
GI	gastrointestinal
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
IEL	intraepithelial lymphocytes
IFNγ	interferon gamma
IL	interleukin
KGF	keratinocyte growth factor
KO	knock-out
LN-5	laminin-5
MMP	matrix metalloproteinase
NEC	necrotizing enterocolitis
PCR	polymerase chain reaction
PG	pyoderma gangrenosum
RECK	reversion-inducing cysteine-rich protein with kazal motifs
RNA	ribonucleic acid
RT-PCR	real-time polymerase chain reaction
SCC	squamous cell cancer
T1D	type I diabetes mellitus
TACE	tumor necrosis factor converting enzyme
TG2	tissue transglutaminase 2
TGFβ1	transforming growth factor beta 1
TIMP	tissue inhibitor of matrix metalloproteinase
TNFα	tumor necrosis factor alpha
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labeling
UC	ulcerative colitis
VEGF	vascular endothelial growth factor

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Abstract

Matrix metalloproteinases (MMPs) comprise a family of 23 zinc-dependent human endopeptidases that can degrade virtually all components of the extracellular matrix (ECM). They are classified into eight subgroups according to their structure and into six subgroups based on their substrate-specificity. MMPs have been implicated in inflammation, tissue destruction, cell migration, arthritis, vascular remodeling, angiogenesis, and tumor growth and invasion. MMPs are inhibited by their natural inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Different MMPs function in the same tasks depending on the tissue or cancer subtype.

I investigated the role of recently discovered MMPs, especially MMPs-19 and -26, in intestinal inflammation, in intestinal and cutaneous wound healing, and in intestinal cancer. Several MMPs and TIMPs were studied to determine their exact location at tissue level and to obtain information on possible functions of MMPs in such tissues and diseases as the healthy intestine, inflammatory bowel disease (IBD), neonatal necrotizing enterocolitis (NEC), pyoderma gangrenosum (PG), and colorectal as well as pancreatic cancers. In latent celiac disease (CD), I attempted to identify markers to predict later onset of CD in children and adolescents. The main methods used were immunohistochemistry, in situ hybridization, and Taqman RT-PCR.

My results show that MMP-26 is important for re-epithelialization in intestinal and cutaneous wound healing. In colon and pancreatic cancers, MMP-26 seems to be a marker of invasive potential, although it is not itself expressed at the invasive front. MMP-21 is upregulated in pancreatic cancer and may be associated with tumor differentiation. MMPs-19 and -28 are associated with normal tissue turnover in the intestine, but they disappear in tumor progression as if they were “protective markers”. MMP-12 is an essential protease in intestinal inflammation and tissue destruction, as seen here in NEC and in previous CD studies. In patients with type 1 diabetes (T1D), MMPs-1, -3, and -12 were upregulated in the intestinal mucosa. Furthermore, MMP-7 was strongly elevated in NEC. In a model of aberrant wound repair, PG, MMPs-8, -9, and –10 and TNFα may promote ECM destruction, while absence of MMP-1 and MMP-26 from keratinocytes retards re-epithelialization.

Based on my results, I suggest MMP-26 to be considered a putative marker for poor prognosis in pancreatic and colon cancer. However, since it functions differently in various tissues and tumor subtypes, this use cannot be generalized. Furthermore, MMP-26 is a beneficial marker for wound healing if expressed by migrating epithelial cells. MMP-12 expression in latent CD patients warrants research in a larger patient population to confirm its role as a specific marker for CD in pathologically indistinct cases. MMP-7 should be considered one of the most crucial proteases in NEC-associated tissue destruction; hence, specific inhibitors of this MMP are worth investigating. In PG, TNFα inhibitors are potential therapeutic agents, as shown already in clinical trials.

In conclusion, studies of several MMPs in specific diseases and in healthy tissues are needed to elucidate their roles at the tissue level. MMPs and TIMPs are not exclusively destructive or reparative in tissues. They seem to function differently in different tissues. To identify selective MMP inhibitors, we must thoroughly understand the MMP profile (degradome) and their functions in various organs not to interfere with normal reparative functions during wound repair or beneficial host-response effects during cancer initiation and growth.

1. Introduction

The human being is as complex as nature itself. If you disturb the balance in one location, the effect is typically not restricted to this region, instead producing unwanted consequences elsewhere. For example, while antibiotic treatment eradicates harmful bacteria, it also disturbs the bacterial balance in the intestine, often resulting in diarrhea. Sometimes exogenous agents are not needed to cause a disease, as in ulcerative colitis, in which man´s endogenous white blood cells attack tissues, causing diarrhea, fever, ulcerations in the intestine, and subsequent bleeding. Chronic inflammation that occurs in conjunction with chronic skin wounds, ulcerative colitis, and pancreatitis can also lead to cancer. Matrix metalloproteinases (MMPs) have been studied intensively after investigations in invasive types of cancer showed upregulation of some previously described MMPs. The results led to rapid development of synthetic MMP inhibitors. However, the outcome of clinical trials using MMP inhibitors has been poor. When some therapeutic advantage was found, the side-effects (e.g. musculoskeletal pain) were too pronounced. Interest in synthetic MMP inhibitors has grown but adequate results are still lacking in the area of cancer research. One reason for the poor results with synthetic MMP inhibitors is the variety of functions of the MMP superfamily and inhibitor therapy generally being started only at later stages of tumor spread in patients with an otherwise poor prognosis. All MMPs do not exist in normal tissue, inflammation, or even cancerous tissue. Their functions may also vary in different diseases or in normal tissue turnover depending on the surrounding extracellular matrix (ECM). Further studies with MMPs will hopefully give us accurate results, enabling specific treatments for cancer and inflammatory conditions to be developed. However, inhibition of MMPs in the intestine or in cancers may cause problems in other tissues or organs. Therefore, simultaneous research of the functions of MMPs in all tissues is important. After these data are attained, targeted inhibitors of MMPs or of their signal transduction cascades can be established and used for curative medicine. The aim of this work was to examine the role and location of recently found MMPs in intestinal inflammation and cancer.

2. Review of the literature

2.1 Structure of intestinal mucosa

The human gastrointestinal (GI) tract consists of a mouth, esophagus, ventricle, small intestine (divided into 3 parts: duodenum, jejunum, and ileum), large intestine (also called colon), and rectum. The intestinal wall in the small intestine and colon comprises four layers: 1) *mucosa*, including epithelium, lamina propria, and muscularis mucosae, 2) *submucosa,* 3) *muscularis externa*, and 4) *serosa* (Figure 1). The inner wall, the lumen, of the small intestine consists of multiple folds and smaller finger-like structures called villi (Figure 2), which multiply the wall area several-fold. Inside the villi are lymphatic vessels, which transport digestive products into larger vessels and finally to the bloodstream. In the large intestine, the inside of the wall is rather flat, but it has a large number of crypts of Lieberkuhn and no lymphatic vessels. The epithelium contains numerous cell types with different functions. In a single layer, *enterocytes* form the majority of the intestinal wall lining (Figures 1 and 2). Enterocytes are responsible for absorption of water, electrolytes, and other dietary substances, but they also secrete enzymes (e.g. disaccharidases to degrade polycarbohydrates). On the apical surface of enterocytes are structures known as microvilli (i.e. brush border), which increase the absorptive area markedly. Another cell type is the *Goblet cell,* which produces and secretes mucus (Figure 2). The secretion of mucus increases towards the end of the small intestine and colon. *Paneth cells* act in host defense and regulate normal bacterial balance. They are located in crypts or mucosal glands (Figure 1). *Enteroendocrine cells* regulate gall bladder and pancreatic activity and gastric motility with secreted, for example, cholecystokinin, secretin, and gastric inhibitory peptide (Figure 1). *M cells* are situated in lymphatic tissues called Peyer´s patches, and they uptake antigens, which are then presented to antigen-presenting cells (APCs). Enterocytes are “born” from stem cells at the bottom of crypts (Figure 1). They then move towards the tip of the villus, where they are shed into the lumen. The intestinal cell population is replaced by new cells every 6 days on average. Enteroendocrine cells last up to 4 weeks (Brandtzaeg et al. 1998; Guyton and Hall 2000; Ross et al. 2003). Underneath the epithelium there is the basement membrane (BM), which is composed of type IV collagen, laminins-1, -2, and -5, nidogen, proteoglycans, tenascin C, and fibronectin. BM components are produced by either epithelial cells or stromal cells. BM regulates cell attachment, growth, and cellular differentiation. The intestinal BM lacks hemidesmosomes (Leivo et al. 1996). The lamina propria is located under the BM. It is highly vascularized, and the major cell types are fibroblasts, myofibroblasts, and leukocytes (MacDonald and Pender 1998). Muscularis mucosae consist of smooth muscle cells that form outer longitudinal and inner circular muscle layers. This structure enables intestinal villi to contract and therefore pump lymphatic fluids to bigger vessels. The submucosa consists of connective tissue and occasional adipocytes. The muscularis externa also has two layers of muscle, and it is responsible for intestinal peristaltic movement, transporting undigestible or later-digested material further down the intestine (Guyton and Hall 2000; Ross et al. 2003).

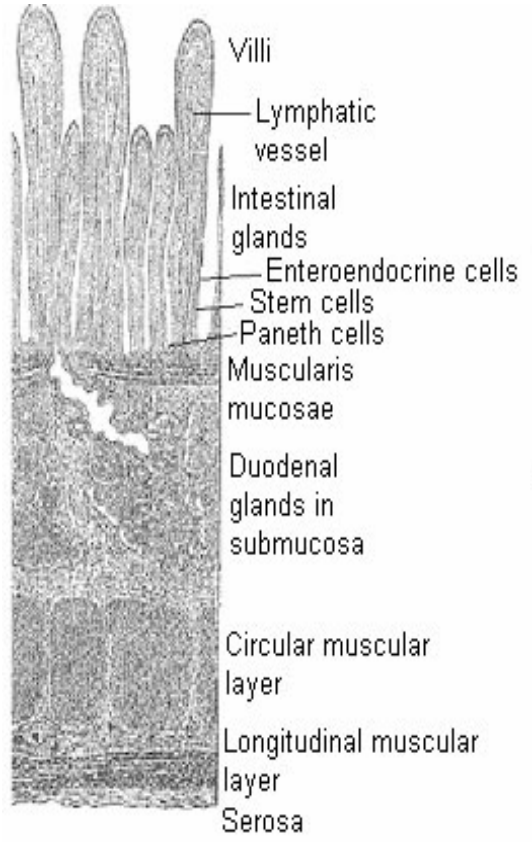

Figure 1. Structure of the wall of the small intestine (modified from Gray 2000).

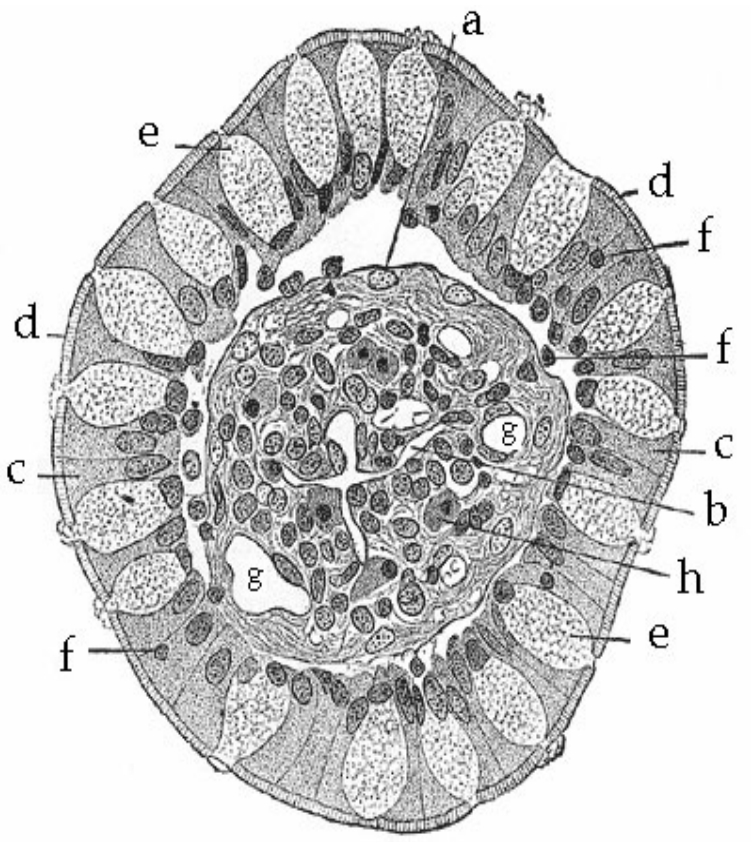

Figure 2. Transverse section of a villus, 350 x magnification. The lumen is located on the outside. a = basement membrane (not correctly attached here), b = lymphatic capillary, c = enterocytes, d = microvilli, e = Goblet cells, f = leukocytes, g = capillary vein, h = cut muscle cell (modified from Gray 2000).

2.2 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) constitute a superfamily of 23 human zinc-dependent endopeptidases involved in tissue remodeling, cell migration, angiogenesis, activation of growth factors, and regulation of inflammation, which characterize cutaneous wound healing and T-cell-mediated gut inflammation and ulceration (Nagase et al. 2006). MMPs are able to degrade most components of the extracellular matrix (ECM) and BMs. However, they also regulate cellular growth factor responses and inflammatory reactions by cleaving and releasing growth factors, cytokines, chemokines, and their receptors, control the activity of defensins, cleave adhesion molecules, and regulate apoptosis (Nagase and Woessner 1999; McCawley and Matrisian 2001; Parks et al. 2004). MMPs are classified according to their structure (Table 1) into eight subgroups or based on their substrate specificity (Table 2) into six subgroups: collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and –9), stromelysins and stromelysin-like MMPs (MMP-3, -10, -11, and -12), matrilysins (MMP-7 and –26), membrane-type MMPs (MMP-14, -15, -16, -17, -24, and –25), and other MMPs (MMP-19, -20, -21, -23, -27, and –28) (Nagase et al. 1999; Uria and López-Otin 2000; Lohi et al. 2001; Ahokas et al. 2002). In addition two other MMPs have been identified, but they are not yet been found in humans: MMP-18 (in

Xenopus laevis), and MMP-22 (in chicken) (Stolov et al. 1996; Yang and Kurkinen 1998). In general, MMPs are produced as zymogens and typically consist of the structural elements of a propeptide, a catalytic domain, a hinge region, and a hemopexin-like domain (Nagase et al. 1999; Table 1). Most MMPs are secreted in the ECM, except the membrane-type MMPs (MT-MMPs) which are bound to the cell membrane. MMPs are activated from proenzymes to active forms in the ECM by themselves, by using other MMPs or other substrates, e.g. plasmin, or intracellularly by furin proteases (Visse and Nagase 2003). MMPs are transcriptionally regulated by cytokines, growth factors, tumor promoters, and cell-cell and cell-matrix interactions. MMPs have been implicated in the pathobiology of rheumatoid arthritis and osteoarthritis (Bresnihan 1999; Ishiguro et al. 1999), tumor growth and metastasis (Vihinen and Kähäri 2002), atherosclerosis (Schonbeck et al. 1997), and chronic cutaneous ulcers (Saarialho-Kere 1998). Moreover, a number of studies suggest that MMPs are important contributors to the breakdown of ECM in disorders characterized by intestinal tissue destruction (Vaalamo et al. 1998; Heuschkel et al. 2001; Salmela et al. 2002). TNFα is one of the most important inducers of MMP protein production (Gan et al. 2001; Nee et al. 2004). MMPs are inhibited by tissue inhibitors of metalloproteinases-1-4 (TIMPs) by forming an inactive complex (Edwards et al. 1996; Gomez et al. 1997; Nagase et al. 2006).

Table 1. All metalloproteinases have a catalytic, zinc-binding domain (Zn) and a pro-peptide that preserves latency. Some contain a furin recognition motif (Fu) that allows intracellular activation by furin-like proteinases. All but MMPs-7, -23, and -26 contain a haemopexin domain that determines substrate specificity. Other domains include the fibronectin-like domains (F) (MMPs-2 and -9) and the vitronectin-like domain (V) (MMP-21). Some MMPs are anchored to the cell surface via a transmembrane component (TM) with a cytoplasmic tail (Cyt) (MMPs-14, -15, -16, and –24) or via a glycosylphosphatidyl inositol (GPI) anchor (MMPs-17 and –25). MMP-23 is structurally unique and contains an N-terminal signal anchor, a cysteine array (CA), and an immunoglobulin-like domain (Ig-like) (modified from Cawston and Wilson 2006).

Group 1	MMP-7, -26	
Group 2	MMP-1, -3, -8, -10, -12, -13, -19, -20, -27	
Group 3	MMP-2, -9	
Group 4	MMP-11, -28	
Group 5	MMP-21	
Group 6	MMP-14, -15, -16, -24	
Group 7	MMP-17, -25	
Group 8	MMP-23	

Table 2. All MMPs and their substrates investigated in this study (modified from Birkedal-Hansen 1995; Lohi et al. 2001; Kerkelä and Saarialho-Kere 2003; Marchenko et al. 2003; Sadowski et al. 2005; Nagase et al. 2006).

MMP	Substrates
Collagenase-1 (MMP-1)	Collagens I, II, III, VII, VIII, X, aggrecan, myelin binding protein, serpins, α2-macroglobulin, perlecan, pro-TNFα, insulin-like growth factor binding protein, α1-proteinase inhibitor, entactin/nidogen, vitronectin, tenascin, fibrinogen, fibronectin, interleukin-1β, monocyte chemoattractant protein-3, protease-activated receptor-1
Collagenase-2 (MMP-8)	Collagens I, II, III, VII, VIII, X, aggrecan, serpins, α2-macroglobulin, fibrinogen, mouse chemokine ligand CXCL
Gelatinase-2 (MMP-9)	Collagens I, IV, V, VII, XI, XIV, XVII, elastin, fibronectin, fibrillin, gelatin, osteonectin, aggrecan, vitronectin, α1-proteinase inhibitor, pro-TNFα, myelin binding protein, insulin-like growth factor binding protein, α2-macroglobulin, plasminogen, TGFβ, decorin, pro-VEGF, fibrin, intercellular adhesion molecule-1, galactin-3, interleukin-1β, interleukin-2Rα
Stromelysin-1 (MMP-3)	Collagens III, IV, V, VII, IX, X, fibronectin, fibrillin, gelatin, aggrecan, laminin-1, nidogen, osteonectin, decorin, α1-proteinase inhibitor, pro-TNFα, myelin binding protein, E-cadherin, β-catenin, insulin-like growth factor binding protein, fibrinogen, vitronectin, tenascin, plasminogen, perlecan, monocyte chemoattractant protein-3, elastin (poorly)
Stromelysin-2 (MMP-10)	Collagens III, IV, V, IX, X, XIV, laminin-5 gamma-2-chain, elastin, fibronectin, gelatin, aggrecan, laminin-1, nidogen
Metalloelastase (MMP-12)	Collagen IV, elastin, fibronectin, laminin-1, gelatin, vitronectin, entactin, proteoglycan, heparan and chondroitin sulfates, pro-TNFα, plasminogen, fibrillin, fibrinogen, α1-proteinase inhibitor
Matrilysin-1 (MMP-7)	Collagen IV, elastin, fibronectin, laminin-1, entactin, aggrecan, vitronectin, versican, nidogen, tenascin, α1-proteinase inhibitor, osteopontin, myelin binding protein, decorin, osteonectin, E-cadherin, plasminogen, β4-integrin, pro-α-defensin, Fas ligand, RANK ligand, pro-TNFα, syndecan-1, heparin-binding EGF
Matrilysin-2 (MMP-26)	Collagen IV, gelatin, fibronectin, fibrin, α1-proteinase inhibitor, β-casein, TNFα-converting enzyme, proMMP-9
MMP-19	Collagen IV, gelatin, laminin-1, nidogen, tenascin, fibrinonectin, aggrecan, cartilage oligomeric matric protein, fibrinogen, laminin-5 gamma-2-chain
MMP-21	α1-antitrypsin
Epilysin (MMP-28)	Casein

2.2.1 MMP-1 and MMP-8 (collagenase-1 and collagenase-2)

MMP-1 (collagenase-1) was the first MMP identified; it was found in the metamorphosing tadpole (Gross and Lapière 1962). It is the major collagenolytic enzyme in human fibroblasts (Overall et al. 1989). MMP-1 is also found in keratinocytes, endothelial cells, monocytes, and macrophages (Pilcher et al. 1998; Saarialho-Kere 1998; Arihiro et al. 2001; Salmela et al. 2004). ProMMP-1 is activated by plasmin, kallikrein, chymase, MMP-3, MMP-7, and MMP-10 (Imai et al. 1995; Suzuki et al. 1995; Nagase and Woessner 1999; Lijnen 2001); it mainly cleaves type III collagen (Nwomeh et al. 1998), but is capable of degrading several other collagen types as well (Birkedal-Hansen 1995; Table 2). MMP-1 is instrumental for keratinocyte migration on type I collagen in healing wounds (Pilcher et al. 1998). MMP-1 is also associated with embryonic development and malignant tumors (McGowan et al. 1994; Stetler-Stevenson et al. 2001). MMP-1 overexpression in differentiating keratinocytes of transgenic mice increased the incidence of chemically induced skin tumors (D´Armiento et al. 1995), possibly due to epidermal hyperproliferative changes augmenting the sensitivity of the skin to carcinogenesis.

MMP-8 (neutrophil collagenase, collagenase-2) is found in polymorphonuclear leukocytes and is stored in granules before being released into the extracellular space (Hasty et al. 1990). It has also been observed in chondrocytes and fibroblast-like cells in the rheumatoid synovial membrane and in endothelial cells, gingival fibroblasts, bronchial epithelial cells, and melanoma cells (Cole et al. 1996; Hanemaaijer et al. 1997; Abe et al. 2001; Giambernardi et al. 2001; Prikk et al. 2001). MMP-8 is activated by plasmin, MMP-3, and MMP-10 (Nagase and Woessner 1999). It mainly cleaves type I collagen, but also types VII, VIII, X, and other ECM proteins (Armstrong and Jude 2002; Figure 4). MMP-8 is the predominant collagenase found in skin wound exudates (Nwomeh et al. 1999). MMP-8 male KO mice have an increased risk for developing chemically induced skin tumors (Balbin et al. 2003), suggesting that MMP-8 has an important role in maintaining inflammatory response induced by carcinogens. On the other hand, expression of MMP-8 correlated with the absence of metastases in an isogenic human breast cancer model (Agarwal et al. 2003).

2.2.2 MMP-9 (92-kDa gelatinase)

MMP-9 is expressed by macrophages, neutrophils, fibroblastic cells, vascular smooth muscle cells, migrating keratinocytes, and osteoclasts (Wucherpfennig et al. 1994; Leppert et al. 1995; Baugh et al. 1999; Arihiro et al. 2001; Saarialho-Kere et al. 2002; Kirkegaard et al. 2004). It is activated by plasmin, trypsin, chymotrypsin, cathepsin G, MMP-3, MMP-7, MMP-10, and MMP-26 (Nakamura et al. 1998; Nagase and Woessner 1999; Lijnen 2001; Marchenko et al. 2001a; McCawley and Matrisian 2001); it mainly cleaves gelatin and type IV collagen, but also other BM components (Birkedal-Hansen 1995; Shipley et al. 1996; Figure 4). MMP-9 has angiostatin-converting enzyme activity (Patterson and Sang 1997). MMP-9 is upregulated by TNFα in a variety of cell types (Van den Steen et al. 2002), particularly during cutaneous wound repair (Scott et al. 2004). TGFβ1 and IL-1β also upregulate MMP-9 expression, at least in keratinocytes (Salo et al. 1994). TIMP-1 is the most potent inhibitor of MMP-9. In MMP-9-deficient mice, vascularization and ossification are delayed (Vu et al. 1998), while wound healing is accelerated (Mohan et al. 2002). These mice also demonstrate reduced skin tumorigenesis, but more aggressive tumors, and reduced pancreatic tumorigenesis (Bergers et al. 2000; Coussens et al. 2000). These results suggest that overexpression of MMP-9 impairs wound healing and to some extent inhibits metastatic activity in tumors.

2.2.3 MMP-3 and MMP-10 (stromelysin-1 and stromelysin-2)

MMP-3 activates proMMPs -1, -3, -7, -8, -9, and -13 (McCawley and Matrisian 2001) and it is itself activated also by plasmin, kallikrein, chymase, and tryptase (Nagase and Woessner 1999; Lijnen 2001). It is expressed in keratinocytes and fibroblasts (Birkedal-Hansen 1993; Saarialho-Kere 1998). MMP-3 can degrade proteoglycans and several other ECM-associated proteins (Murphy et al. 1991; Table 2). In MMP-3 KO mice, chemically induced SCCs behave more aggressively (McCawley et al. 2004), suggesting that MMP-3 acts in an anti-tumorigenic manner in normal tissue and tumors. These mice also have difficulties in wound contraction (Bullard et al. 1999), implying a malfunction in myofibroblasts.

MMP–10 activates proMMPs -1, -2, -7, -8, and -9 (Nicholson et al. 1989; Windsor et al. 1993; Knäuper et al. 1996; Nakamura et al. 1998) and it is itself activated by plasmin, elastase, and cathepsin G (Nagase and Woessner 1999; Lijnen 2001). It degrades proteoglycans, globular type IV and IX collagens, laminin-1, fibronectin, and other ECM-associated proteins (McCawley and Matrisian 2001; Table 2). MMP-10 is expressed by keratinocytes in vivo and in culture (Rechardt et al. 2000). MMP-10 overexpression in transgenic mice leads to disorganization of the migrating tip in wounds (Krampert et al. 2004). Hence, overexpression may lead to impaired wound healing, whereas normal regulation promotes epithelial repair.

2.2.4 MMP-12 (human metalloelastase)

MMP-12 is expressed in macrophages and it may help them to migrate to sites of inflammation by degrading BMs of endothelial cells and of the mucosal epithelium. MMP-12 may also regulate inflammatory response since it can activate TNFα (Chandler et al. 1996). TNFα, IL-1β, plasmin, M-CSF, VEGF, and PDGF-BB upregulate MMP-12 in macrophages in vitro, but MMP-12 is inhibited by TGFβ and α1-antitrypsin via a reduction in plasmin activity (Cornelius et al. 1998; Feinberg et al. 2000; Churg et al. 2007). MMP-12 is also expressed in hypertrophic chondrocytes and osteoclasts (Kerkelä et al. 2001; Hou et al. 2004). The substrate specificity of MMP-12 includes elastin, type IV collagen, fibronectin, laminin-1, entactin, and proteoglycans, but MMP-12 is unable to degrade interstitial collagens or gelatin (Chandler et al. 1996; Gronski et al. 1997; Table 2). MMP-12 KO mice have a lower risk for having smoking-induced emphysema (Hautamäki et al. 1997) and a higher risk for lung cancer metastases than their wild-type counterparts (Houghton et al. 2006). This may be due to MMP-12 being able to cleave plasminogen into angiostatin, which inhibits angiogenesis and tumor growth (Dong et al. 1997; Cornelius et al. 1998).

2.2.5 MMP-7 and MMP-26 (matrilysin-1 and matrilysin-2)

MMP-7 is expressed constitutively by normal exocrine glands (Saarialho-Kere et al. 1995). It is activated by plasmin and MMP-3 (Nagase and Woessner 1999; Lijnen 2001), cleaves several BM-associated proteins (Sternlicht and Werb 2001; Table 2), and activates α-defensins (Lopez-Boado et al. 2000). MMP-7 is upregulated in several tumors, including breast, lung, upper respiratory tract, skin, stomach, and colon cancers (Basset et al. 1990; McDonnell et al. 1991; Muller et al. 1991; Karelina et al. 1994; Newell et al. 1994), as well as in injured intestinal epithelium (Saarialho-Kere et al. 1995), as seen in rectal cancer treated preoperatively with radiotherapy (Kumar et al. 2002). However, it is able to inhibit angiogenesis in tumors by generating angiostatin (Patterson and Sang 1997; Pozzi et al. 2000). MMP-7 KO mice have reduced innate intestinal immunity, impaired mucosal re-epithelization in the airways, and reduced intestinal tumorigenesis (Wilson et al. 1997, 1999; Dunsmore et al. 1998). Hence, MMP-7 could act physiologically as a mediator of antibacterial activity, epithelial repair, and proliferation.

MMP-26 (endometase/matrilysin-2) was cloned simultaneously by three different laboratories, from fetal (de Coignac et al. 2000), placental (Uria and López-Otin 2000), and endometrial tumor cDNAs (Park et al. 2000). It is the smallest MMP family member, with a molecular mass of 28 kDa (Park et al. 2000), and it can auto-activate (Marchenko et al.

2003). In vitro MMP-26 degrades, e.g., fibronectin, vitronectin, fibrinogen, type IV collagen, gelatin, and α1-proteinase inhibitor and is able to activate proMMP-9, but cannot cleave type I collagen, laminin, elastin, or lactoferrin (Uria and López-Otin 2000; de Coignac et al. 2000; Park et al. 2000; Marchenko et al. 2001a; Table 2). TIMP-4 is the most potent inhibitor of MMP-26 (Zhang et al. 2002). MMP-26 expression has been found in several cancer cell lines in culture. A significant level of expression in healthy tissues has been recorded by Northern blot analysis in the kidney and placenta, and by RT-PCR in cancers of the endometrium, lung, prostate, and mammary gland (de Coignac et al. 2000; Park et al. 2000; Uria and López-Otin 2000; Marchenko et al. 2001a). In vivo data on human MMP-26 protein are limited to normal human cytotrophoblasts (Zhang et al. 2002), normal endometrial glands (Isaka et al. 2003), prostate cancer (Zhao et al. 2003), esophageal cancer (Ahokas et al. 2006), and migrating keratinocytes (Ahokas et al. 2005). Therefore, it has been suggested to be involved in implantation and could be a target enzyme in the treatment of cancer and other pathological conditions. There are no KO studies on MMP-26 since this gene does not exist in rodents (Puente and Lopez-Otin 2004).

2.2.6 MMP-19

MMP-19 was originally cloned from the mammary gland (Cossins et al. 1996) and liver (Pendas et al. 1997). It has also been isolated from the inflamed rheumatoid synovium as an autoantigen (RASI) (Sedlacek et al. 1998). MMP-19 mRNA can be detected by Northern hybridization in many tissues, including the placenta, lung, pancreas, ovary, spleen, and intestine (Pendas et al. 1997), as well as in acutely inflamed synovial tissue, especially in capillary endothelial cells, suggesting a role in angiogenesis (Kolb et al. 1999). MMP-19 has also been found in proliferating keratinocytes, fibroblasts, myoepithelial, and smooth muscle cells, and in association with the cell surface of myeloid cells (Kolb et al. 1999; Djonov et al. 2001; Mauch et al. 2002; Suomela et al. 2003). MMP-19 is able to degrade in vitro, e.g., type IV collagen, laminin-1, nidogen, tenascin-C, fibronectin, and type I gelatin, but is not capable of activating any proMMPs (Stracke et al. 2000a, 2000b). Unlike classical MMPs, MMP-19 is found in the resting mammary gland and its benign lesions, but progression towards an invasive phenotype and neoplastic dedifferentiation lead to its disappearence from tumor cells and blood vessels (Djonov et al. 2001), suggesting that it is some kind of “protective marker”. This has also been observed in cutaneous SCC (Impola et al. 2003). MMP-19 KO mice have an increased risk for developing diet-induced obesity, but a lower risk for having chemically induced cancer (Pendas et al. 2004). Thus, MMP-19 could be involved in cell proliferation and normal tissue turnover.

2.2.7 MMP-21

MMP-21 was originally cloned from human placenta cDNA (Ahokas et al. 2002). The only known physiological substrate for MMP-21 is α1-antitrypsin, and furin is its putative activator (Ahokas et al. 2002; Marchenko et al. 2003). It has important roles during fetal development and in cancer biology (Ahokas et al. 2003, 2006; Marchenko et al. 2003). MMP-21 is regulated at least in keratinocytes by TGFβ1 and is present at the invasive front of cutaneous and esophageal SCCs but has not yet been observed in dysplastic cells (Ahokas et al. 2003, 2006). MMP-21 can also be expressed by macrophages and fibroblasts in vivo and in vitro (Skoog et al. 2006) and by neutrophils in vivo (Ahokas et al. 2002, 2006).

2.2.8 MMP-28 (epilysin)

MMP-28 (epilysin) is one of the most recently cloned human MMPs. It is a 59-kDa protein (Lohi et al. 2001; Illman et al. 2001) and is most closely related to MMP-19. MMP-28 is activated intracellularly by furin (Visse and Nagase 2003). MMP-28 is known to degrade casein (Lohi et al. 2001). The highest levels of MMP-28 mRNA are found in the skin in basal and suprabasal keratinocytes, in the developing germ cells of the testis (Lohi et al. 2001), and in bone, kidney (Bernal et al. 2005), and lung (Marchenko et al. 2001b). MMP-28 is also expressed in several carcinomas at the tissue level, including pancreatic, colon, ovarian, lung, and prostate carcinomas (Marchenko and Strongin 2001b). It is associated with proliferative cells in epithelial wound repair (Lohi et al. 2001; Saarialho-Kere et al. 2002), but not in psoriatic, resting, or migrating epithelium (Saarialho-Kere et al. 2002; Suomela et al. 2003).

2.3 Natural inhibitors of MMPs - tissue inhibitors of metalloproteinases (TIMPs)

MMP activity is modulated by their natural tissue inhibitors, TIMPs-1-4 (Edwards et al. 1996; Gomez et al. 1997). TIMPs inhibit the activity of MMPs by forming stoichiometric 1:1 complexes. TIMPs´ structure is a “wedge-like” shape that fits into the active site of MMPs like into a slot, and chelates MMPs´ zinc atom (Nagase et al. 2006). Almost all MMPs are inhibited by all four TIMPs. However, TIMPs also have functions independent of their MMP-inhibitory effects: they can stimulate cell growth and proliferation, inhibit angiogenesis, and promote or suppress apoptosis (Mannello and Gazzanelli 2001). Their diminished or increased expression has been reported in various cancers depending on the tumor type (Salmela et al. 2001; Ahonen et al. 2003). In general, TIMPs inhibit tumorigenesis, but TIMPs-1 and -4 have been found to promote metastasis (Baker et al. 2002).

2.3.1 TIMP-1

TIMP-1 was the first TIMP found in culture medium of human fibroblasts in 1975 (Bauer et al. 1975). The molecular weight of this protein is 28.5 kDa (Stricklin and Welgus 1983). TIMP-1 inhibits almost all MMPs, but it is not capable of properly inhibiting MMPs-14, -15, -16, -19, and -24 (Baker et al. 2002). TIMP-1 is expressed by skin fibroblasts, and inflammatory cells, fibroblasts and vascular smooth muscle cells in the inflamed intestine (Saarialho-Kere et al. 1996; Vaalamo et al. 1998; Arihiro et al. 2001). TNFα downregulates the expression of TIMP-1 (Yao et al, 1997). TIMP-1-deficient mice live normally, but have a shorter lifespan than wild-type mice (Nothnick 2001) and also are more resistant to Pseudomonas aeruginosa infection (Coussens et al. 2001). TIMP-1 overexpression in transgenic mice leads to a reduced risk for mammary or liver cancer (Yamazaki et al. 2004; Rhee et al. 2004). In human breast epithelial cells, TIMP-1 has an anti-apoptotic effect (Li et al. 1999).

2.3.2 TIMP-3

TIMP-3 was cloned from the human placenta cDNA library in 1994 (Apte et al. 1994). The molecular weight of TIMP-3 protein is 27 kDa (Apte et al. 1995). TIMP-3 inhibits all MMPs and TACE (Mannello and Gazzanelli 2001). While the other TIMPs are soluble, TIMP-3 is ECM-bound (Baker et al. 2002). TIMP-3 is expressed in keratinocytes and fibroblasts (Vaalamo et al. 1999), and in a variety of cancers, including esophageal, colorectal, endometrial, prostatic, and breast cancers (Karan et al. 2003; Tunuguntla et al. 2003; Curran et al. 2004; Miyazaki et al. 2004; Darnton et al. 2005; Mylona et al. 2006). TIMP-3 deficiency in mice disrupts matrix homeostasis and causes spontaneous left ventricular dilation, cardiomyocyte hypertrophy, and contractile dysfunction (Fedak et al. 2004). TIMP-3-deficient mice suffer from lung emphysema, which is probably caused by an imbalance in the MMP/TIMP ratio, followed by overwhelming collagenolysis (Leco et al. 2001). These mice also show accelerated mammary epithelial apoptosis (Fata et al. 2001).

2.3.3 TIMP-4

TIMP-4 was cloned from the human heart library in 1996, and it is 51% identical to TIMPs-2 and -3. The molecular weight of TIMP-4 protein is 22 kDa (Greene et al. 1996). It is anti-inflammatory, can induce apoptotic cell death in transformed cells (Mannello and Gazzanelli 2001), and inhibits all MMPs, particularly MMP-26 (Radomski et al. 2002). On the other hand TIMP-4 inhibits apoptosis in human breast cancer cells (Jiang et al. 2001). TIMP-4 expression is upregulated also in dysplastic changes in prostatic tissue, but downregulated in invasive cancer (Lee et al. 2006). TIMP-4 is upregulated in cervical cancer, ovarian cancer, invasive endometrial cancer and in ductal in situ breast cancer, and it often colocalizes with MMP-26 (Tunuguntla et el 2003; Zhao et al. 2004; Lizarraga et al. 2005; Ripley et al. 2006). TIMP-4 mRNA is also expressed in normal endometrial stroma and is induced by estrogen (Pilka et al. 2006).

2.3.4 Other endogenous MMP inhibitors

Several other proteins have also been reported to inhibit MMP activity. MMP-2 is inhibited by RECK, β-amyloid precursor protein, procollagen C-proteinase enhancer protein, thrombospondin-1 and 2, and chlorotoxin (Mott et al. 2000; Oh et al. 2001; Egeblad and Werb 2002; Deshane et al. 2003; Higashi and Miyazaki 2003). RECK, a membrane-bound GPI-anchored glycoprotein, also inhibits MMP-9 and MMP-14 (Oh et al. 2001). RECK KO mice have deficient vascular development and die before birth in utero (Oh et al. 2001). α2-macroglobulin is produced mainly by hepatocytes, but also by other cell types, e.g. macrophages (Baker et al. 2002). It is known to inhibit almost all endoproteases, including MMP-13 (Nie et al. 2007). The mechanisms of MMP inhibition by these proteins are yet to be discovered.

2.4. MMPs in intestinal inflammation

2.4.1 Inflammatory bowel disease and other colitides

Inflammatory bowel disease (IBD) comprises two different diseases, ulcerative colitis (UC) and Crohn's disease (CrD). IBD is a chronic, relapsing condition with inflammation and tissue remodeling of the GI tract. The main symptoms are abdominal pain, diarrhea, rectal bleeding, and fever. *UC* affects only the rectum and colon, hence the name colitis. The inflammatory process is limited to the mucosa and is histologically characterized by the presence of crypt abscesses and ulcerations (Podolsky 1991; Farrell and Peppercorn 2002). *CrD* may affect any region of the GI tract, from the mouth to the anal canal. The inflammatory process can extend throughout the intestinal wall (transmural), narrowing the intestinal lumen, and form fistulae. Histologically, CrD is characterized by the formation of granulomas and fibrosis (Shanahan 2002). Both CrD and UC can have extraintestinal manifestations and result in susceptibilities to other diseases, such as arthritis, spondylitis, sacroilitis, osteoporosis, erythema nodosum, pyoderma gangrenosum, aphtous ulcers, episcleritis, primary sclerosing cholangitis, gallstones, thromboembolism, perimyocarditis, asthma, and multiple sclerosis (Rothfuss et al. 2006). IBD is usually treated with corticosteroids, and this may lead to diminished bone mineral density (Silvennoinen et al. 1995). The etiology of IBD remains unclear, although environmental, microbiological, immunological, and genetic factors have been implicated in the pathogenesis of the disease. Smoking seems to increase the risk in having familial CrD at a younger age and to reduce the risk of having UC at an older age (Tuvlin et al. 2007). Appendectomy and the use of contraceptives also increase the risk of IBD (Corrao et al. 1998; Loftus 2004). In Japanese studies, HLA-DR2 has been implicated in UC, whereas HLA-DR3 has been suggested to be involved in CrD (Toyoda et al. 1993). A CrD susceptibility gene, NOD2/CARD15, has been detected in chromosome 16, but chromosomes 1, 3, 4, 5 (OCTN1 and 2), 6, 7 (NOD1), 10 (DLG5), 12, 14, 19, and X have also been suggested to be linked to CrD (Hampe et al. 1999; Mathew and Lewis 2004; Peltekova et al. 2004; McGovern et al. 2005). *NOD2/CARD15* mutations have been found in 1.2-4.3% of healthy controls, suggesting that other risk factors besides a single gene mutation are needed for the onset of CrD (Hugot et al. 2007). In sibling studies, the risk of having CrD is 37-58% in monozygotic and 0-7% in heterozygotic twins, with the respective figures for UC being 10-18% and 3-5% (Orholm et al. 2000; Vermeire and Rutgeerts 2005). According to the currently accepted hypothesis, both UC and CrD result from a malfunction of the autonomic nervous system and a dysregulated response of the intestinal immune system towards intraluminal antigens of bacterial origin in genetically predisposed patients. This leads to the activation and release of several factors, including cytokines, nitric oxide, eicosanoids, and proteolytic enzymes, which initiate a cascade of events resulting in intestinal injury (Fiocchi 1997; Podolsky 2002; Taylor and Keely 2007).

Several MMPs have been implicated in the pathobiology of IBD. MMP-9, primarily an inflammatory cell-derived gelatinase, has been shown to be a major factor in adult intestinal tissue destruction and inflammation (Baugh et al. 1999; Tarlton et al. 2000), and its increased expression in the ECM of IBD lesions correlates with the severity of inflammation (Gao et al. 2005). In MMP-9 KO mice, the severity of colonic inflammation is reduced (Castaneda et al. 2005). MMP-1 is involved in mucosal destruction through degradation of several collagen types (Chandler et al. 1997), and its mRNA is found in granulation tissue (Saarialho-Kere et al. 1996) and in inflammatory cells and fibroblasts

(Arihiro et al. 2001). It has been associated with tissue destruction also in experimental IBD models (Pender et al. 1998). In ischemic colitis, stromal cells and migrating enterocytes bordering intestinal ulcers express MMP-1 mRNA (Salmela et al. 2004). Also MMPs-3, -7, -10, and -12 have been suggested to be the main MMPs in intestinal inflammation (Vaalamo et al. 1998; Salmela et al. 2002; Matsuno et al. 2003). In an experimental model of IBD, MMP-10 was expressed in areas with the most severe injury (Salmela et al. 2002). Therefore, it is clear that inhibition of MMP-10 reduces the severity of inflammation in IBD (Kobayashi et al. 2006). MMP-12 is upregulated in experimental models of T-cell-mediated tissue injury of the intestine (Salmela et al. 2001, 2002), and animal studies suggest that MMP-12 may partly contribute to cryptal hyperplasia (Li CK et al. 2004). The ratios of MMP-1/TIMP-1 and MMP-3/TIMP-1 have been shown to increase in the inflamed intestine (von Lampe et al. 2000).

Necrotizing enterocolitis (NEC) is the most common gastrointestinal disease of premature infants, with an overall mortality rate of 20-30% (Neu 1996; Dahms 2001). The symptoms vary dramatically, ranging from benign gastrointestinal disturbance to intestinal gangrene and perforation, sepsis, and shock (Dahms 2001; Kliegman and Fanaroff 1984). In X-rays, visible air bubbles may be present inside the intestinal wall. The only treatment to date is surgical removal of the necrotic part of the colon and the small intestine. Histologically, coagulative and hemorrhagic necrosis, limited to the mucosa in the early stages, but at least focally transmural when the surgical removal occurs, is observed in the ileum and cecum (Dahms 2001). The ultimate pathobiology of NEC is unknown, but prematurity, onset of enteral feeding, and infection have been identified as predisposing factors. Mixed intestinal bacteria are often visible in the lumen or the necrotic superficial mucosa (Hsueh et al. 2003). TIMP-1 mRNA is increased as is MMP-3 mRNA in myofibroblasts of NEC patients in vivo, but MMP-1, -2, -9, and TIMP-2 amounts are low (Pender et al. 2003). TNFα is often connected to inflammatory processes, and it is upregulated in NEC tissue (Pender et al. 2003). Hence, in NEC, which has severe inflammation, TNFα is upregulated by and through tissue destruction. MMP-3 upregulation in NEC results from tissue destruction and formation of new stroma by myofibroblasts. TIMP-1 is involved in reducing MMP overexpression.

2.4.2 Celiac disease

The estimated prevalence of celiac disease (CD) in Finnish children is 1.0-1.7% (Mäki et al. 2003). CD is caused by intolerance to gliadins and other prolamins, which presents as a Th-1 type immune response and characteristic enteropathy in genetically susceptible individuals (Trier 1991; Farrell and Kelly 2002). The exposure of the ileal mucosa to such proteins increases the number of intraepithelial cytotoxic T cells and T helper cells in the lamina propria, and particularly the increase in gamma/delta T cells is associated with the presence of CD (Savilahti et al. 1997; Järvinen et al. 2003). Clinical manifestations vary widely, from practically nonexistent to severe, and consist of gastrointestinal, nutritional, and dermatologic symptoms (Farrell and Kelly 2002; Haapalahti et al. 2005). Potential patients are screened by testing blood for anti-tissue transglutaminase (TG2) and anti-endomysium (EMA) antibodies. These two antibodies share their target antigens. In active CD, these specific antibodies are directed against TG2 and might be important in the formation of mucosal lesions, as they inhibit epithelial cell differentiation in a small-bowel mucosal crypt villus axis model (Halttunen and Mäki 1999; Sollid 2002). Both TG2 and EMA antibody tests are highly specific for overt CD, in which villous atrophy, cryptal

proliferation, and elevated numbers of intraepithelial lymphocytes (IEL) are seen (Walker-Smith et al. 1990; Farrell and Kelly 2002). These changes are driven by elevated expression of IFNα, which supports Th1 response to tissue injury and can enhance IFNγ and TNFα production (Monteleone et al. 2001). When there is no villous atrophy, but only IELs, in duodenal biopsies, the CD diagnosis cannot be confirmed. In some of these patients, CD will manifest at a later date (Ferguson et al. 1993). Although the presence of HLA class II heterodimer HLA-DQ2 or HLA-DQ8 implies a genetic predisposition for CD, it is of limited value as a prognostic marker. Thus, to avoid repeated biopsies and unnecessary dietary restrictions, new specific markers are needed for the clinical judgment of patients with positive screening tests for CD, but with no or only slight mucosal changes.

MMPs have been implicated in the pathobiology of celiac intestinal lesions. Subepithelial macrophages and fibroblasts express MMP-1 and MMP-3 in the celiac intestine, and a gluten-free diet has been demonstrated to diminish the number of these cells (Daum et al. 1999). In cultured cells, MMPs-2 and -9 are also upregulated, but expression of TIMPs-1 and -2 remains unchanged (Pender et al. 1997). Metalloelastase (MMP-12) is abundantly expressed by subepithelial macrophages in the celiac intestine of adult dermatitis herpetiformis patients, while MMP-7, -10, and -13-positive cells are not found in their intestinal samples (Salmela et al. 2001). Thus, morphological alterations of the duodenal/jejunal mucosa in CD may be associated with increases in the concentrations and activities of particular MMPs derived from immunological processes induced by gliadin in susceptible individuals.

2.5 MMPs in cancers of the gastrointestinal tract

Coexpression of several members of the MMP family is characteristic of human malignant tumors (van Kempen et al. 2002). Classical MMPs play an important role at all stages of tumorigenesis; they enhance tumor-induced angiogenesis, release growth factors from the matrix or cleave their receptors, regulate apoptosis, and break down the matrix and BMs to allow tumor cell invasion and metastatic spread (McCawley and Matrisian 2000; Liotta and Cohn 2001; Stetler-Stevenson and Yu 2001). Paradoxically, several MMPs (MMPs-3, -7, -9, and –12) can release angiogenic inhibitors, such as angiostatin and endostatin (Cornelius et al. 1998; Nyberg et al. 2003), and thus their peritumoral expression may serve to limit cancer growth, probably depending on the tumor type. MMP-9 is suggested to be associated with esophageal carcinogenesis in Barrett´s esophagus (Herszenyi et al. 2007). In esophageal squamous cell carcinoma (ESCC), MMP-9 is associated with lymph node metastasis and poor prognosis along with MMPs-1, -7, and -13 (Murray et al. 1998a; Gu et al. 2005), while TIMP-3 expression is downregulated dramatically and the reduction of TIMP-3 is associated with invasiveness and poor survival (Darnton et al. 2005; Faried et al. 2006). High tissue levels of MMPs-1, -2, -9, -14, and TIMP-1 are associated with poor survival in gastric carcinoma (Sier et al. 1996; Bando et al. 1998; Murray et al. 1998b; Yoshikawa et al. 2001). ProMMP-2 activation is reduced by TIMP-3 in linitis plastica-type gastric cancer (Yokoyama et al. 2004). MMPs-2, -9, -14, and TIMP-2 are highly expressed in hepatocellular carcinoma, whereas MMPs-7, -15, and TIMP-1 are found in low concentrations (Ogasawara et al. 2005). MMPs-2 and -9 are elevated in stromal cells in hepatocellular carcinoma of liver transplant patients (Zhang et al. 2006).

2.5.1 Colorectal cancer

Colorectal cancer is the third most common cancer in Finland (Finnish Cancer Registry, www.cancerregistry.fi). Risk factors include a low-fiber high-fat diet, smoking, high alcohol consumption, obesity, and diabetes mellitus (Weitz et al. 2005). Colorectal cancer is generally a disease of older people. In most cases, it arises from a single adenomatous polyp, and therefore, is considered to represent adenocarcinoma (Winawer et al. 1993; Nozoe et al. 2000). However, new subtypes of adenocarcinoma precursors have been discovered: serrated adenoma and sessile serrated adenoma, which use different pathways to develop into fulminant adenocarcinoma (Makinen 2007). Almost 98% of colorectal tumors are adenocarcinomas. Less than 2% of the colorectal tumors are carcinoid tumors, which are of endocrine cell origin. Also adenosquamous cell tumors occur in the rectal area with cutaneous features, but these are rare, and lymphomas, which mainly locate themselves in the small intestine (Crawford 1999). About 70% of colorectal cancers are considered to be sporadic (de la Chapelle 2004). Colorectal cancers of hereditary origin account for up to 30% of all cases: hereditary nonpolypotic colorectal cancer (~4%), familial adenomatous polyposis (0.5-1.5%), other polyposis syndromes (<1%), and inherited susceptibility (20-25%). Inherited susceptibility means that patients have positive family history, but do not meet the criteria for other groups (Bisgaard et al. 1994; Aaltonen et al. 1998; Salovaara et al. 2000; de la Chapelle 2004; Burt and Neklason 2005). Mutations in adenomatous polyposis of the colon (APC)- or TP53-genes or *k-ras* mutations are important contributing factors (Bos et al. 1987; Baker et al. 1990; Powell et al. 1992; Jernvall et al. 1997). About half of the cases are diagnosed when a tumor is local and the other half when it is more spread. The 5-year survival is 75-90% if the tumor is diagnosed as Dukes class A or B (local tumor), 40-50% if Dukes C (lymph node metastasis), and 4% if Dukes D (distant metastasis) (Cohen et al. 1991). Increased inflammation at the invasive front is associated with better survival in Dukes A and B class tumors (Klintrup et al. 2005). Better prognosis is also established in female patients with a low amount of 17-beta-hydroxysteroid dehydrogenase type 2 in their colorectal Dukes A, B, and C class tumors (Oduwole et al. 2003).

MMP-1 expression in colorectal cancer is a marker of metastatic activity and poor prognosis (Zucker and Vacirca 2004). MMP-2 is elevated in colon cancer in stromal cells, but consistent data on aggressiveness have not yet been presented (Zucker and Vacirca 2004). MMP-3 is elevated in colorectal cancers, probably due to MMP-9-induced plasmin activation (Inuzuka et al. 2000). MMP-7 is expressed in 90% of colonic adenocarcinomas, and it is associated with tumor growth and de-differentiation (Newell et al. 1994; Wilson et al. 1997). It has also been seen in cultured endothelial cells, suggesting a direct role in inducing angiogenesis (Huo et al. 2002). MMP-9 is associated with early relapse and poor survival (Zeng et al. 1996). MMP-12 is associated with reduction in tumor size, increased survival, and inhibition of neovascularization (Yang W et al. 2001; Zucker and Vacirca 2004). MMP-13 expression is associated with poor survival (Leeman et al. 2002). MMP-14 expression is increased in higher stage tumors (Sardinha et al. 2000). TIMP-1 is increased in circulating blood in colorectal cancer and is associated with poor prognosis and metastatic activity (Yukawa et al. 2001). TIMPs-1 and -2 are more often found in peritumoral cells than in actual carcinoma cells (Zucker and Vacirca 2004).

2.5.2 Pancreatic carcinoma

Pancreatic cancer is one of the most lethal types of cancer; the 5-year survival rate of pancreatic adenocarcinoma is under 5% (Gudjonsson 1987). In Finland, it was the third leading cause of cancer death after lung and prostate cancer in men, and after breast and lung cancer in women in the year 2003 (Finnish Cancer Registry 2003). Other types of pancreatic cancers (15% of all malignant tumors) include insulinoma, gastrinoma, and carcinoid tumor. Pancreatic cancer is notorious for its late presentation, early and aggressive local invasion, and metastatic potential (Duffy et al. 2003). The diagnosis is confirmed using ultrasound combined with biopsy as well as computer tomography and tumor markers such as CA 19-9. If metastases are not found, the Whipple operation (pancreaticoduodenectomy) is performed to remove the tumor. If metastases are detected, palliative treatments, such as biliary bypass operation or radiotherapy, are offered. The results of treatment with cytostatics have been poor (Duffy et al. 2003). A known risk factor is smoking; smokers have a 2- to 3-fold higher risk than nonsmokers (Boyle et al. 1996). Patients with chronic pancreatitis also have an increased risk for developing pancreatic carcinoma (Ekbom et al. 1994; Bansal et al. 1995).

Many studies on pancreatic cancer have found that increased MMP expression correlates with poorer prognosis, short survival time, or presence of local invasion or distant metastases (Garcea et al. 2005). Particularly MMP-7 overexpression is considered a metastatic and prognostic marker (Yamamoto et al. 2001; Li et al. 2005). Furthermore, MMP-2 and -9 expression in primary tumors is associated with invasiveness and liver metastases in pancreatic carcinomas (Yang X et al. 2001; Matsuyama et al. 2002), and MMP-1 and MMP-12 with poor prognosis (Ito et al. 1999; Balaz et al. 2002). The role of MMP-12 in inhibiting angiogenesis is not crucial for cancer cell survival in pancreatic cancer because of the type of tumor spreading. Pancreatic cancer progresses as single cell extensions rather than as an ever-widening solid tumor. Also MMPs-14 and -15, but not MMP-16, are elevated in pancreatic cancer (Imamura et al. 1998; Ellenrieder et al. 2000) and have been proposed to have a role in desmoplastic reaction. MMP inhibition by TIMP-1 antisense gene transfection or by using synthetic protease inhibitors reduces the invasive potential of pancreatic cancer cells in experimental models (Zervox et al. 2000; Bloomston et al. 2005). Pancreatic cancer overexpresses growth factors, such as EGF, VEGF, and FGF, and cytokines, such as TNFα, TGFβ, and interleukins 1, 6, and 8 (Yamanaka et al. 1993; Korc 1998; Saito et al. 1998; Kleeff et al. 1999; Luo et al. 2001). Laminin-5 (LN-5) expression is elevated in circulating blood and at the tissue level in patients with metastatic pancreatic adenocarcinoma (Tani et al. 1997; Fukushima et al. 2001; Katayama et al. 2005).

2.6 Wound healing

2.6.1 Normal wound healing of the intestine and skin

In normal wound healing, epithelial disruption is followed by systematic procedures to cover the wound bed and restore normal organization (Martin 1997; Podolsky 1999). The wound repair can be divided into four overlapping phases: coagulation, inflammation, migration-proliferation (including matrix deposition), and remodeling (Falanga 2005; Figure 3). If the wound is superficial, the BM is either intact or newly formed by epithelial cells (enterocytes in the intestine and keratinocytes in the skin) and stromal cells, and the adjacent epithelial cells become flattened and start to migrate over the injured area until it is fully covered with epithelial cells (Hudspeth 1975). If the wound is deeper and the stromal compartment is also involved, often vascular structures are damaged and bleeding occurs. This leads to release of growth factors and cytokines, blood clotting involving thrombocyte aggregation, fibrin formation to stop bleeding, and migration of endothelial cells that form new microvessels (Goss et al. 1992; Singer and Clark 1999). Inflammatory cells, neutrophils, and macrophages also migrate to the region, and subepithelial myoepithelial cells assist in contracting the wound edges closer to each other. The healing process includes fibrinolysis after the bleeding ceases, the epithelium is intact, and the newly formed vascular structure is ready. These different events occur in a spatially and temporally regulated manner in normal wound healing. The wound healing process starts immediately after the injury and the elapsed time for sufficient wound healing ranges in intestinal superficial wounds from hours to days and in deeper cutaneous wounds from a few days to 1-2 weeks or even months. In superficial wounds, the only indication of injury may be the memory of it. In deeper skin wounds, a visible scar will remain because of fibrosis. In the intestine, scarring is rare (Goss et al. 1992).

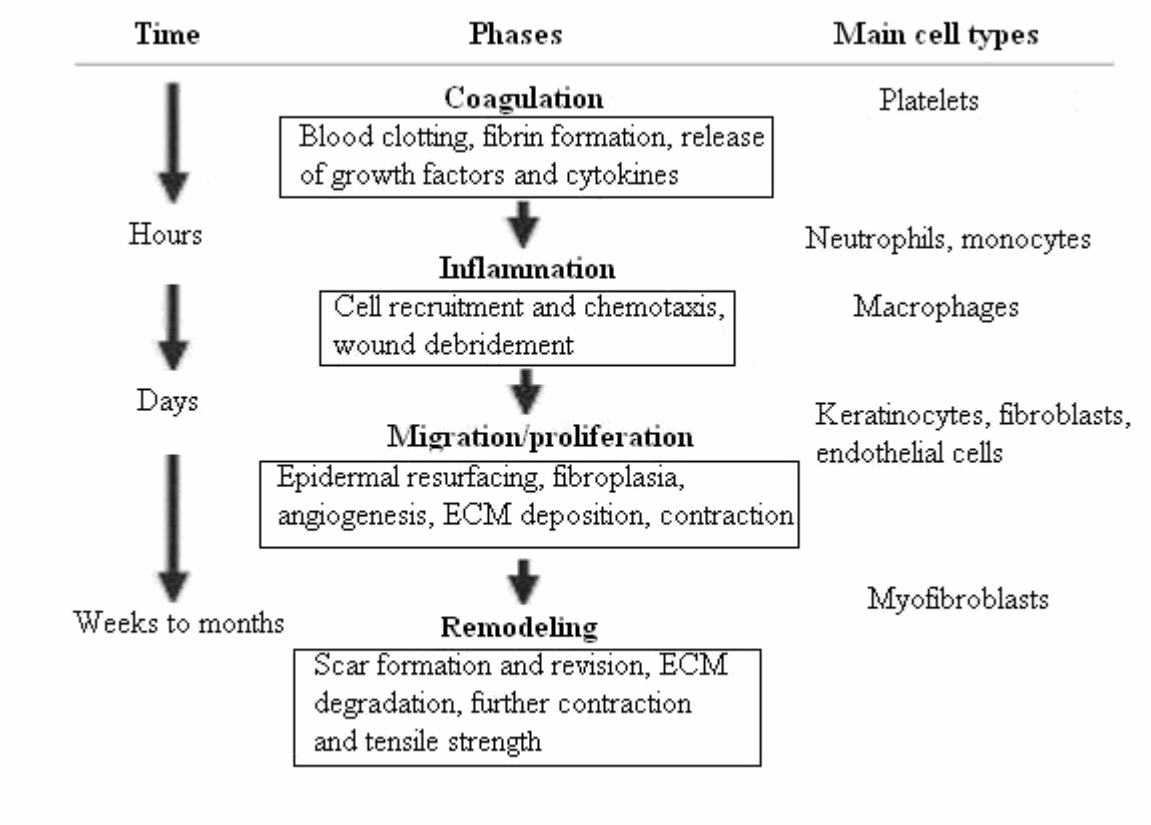

Figure 3. Four phases of the healing process in normally healing cutaneous wounds (modified from Falanga 2005).

In this healing process, cells interact with different cytokines and ECM substrates. Migration of epithelial cells is more rapid on a type I collagen wound bed, which is the main collagen type in the ECM, than on newly formed BM consisting of type IV collagen, laminin, and fibronectin (Basson 2001). Migrating epithelial cells express cytoplasmic LN-5, which is used as a marker of migrating cells and is cleaved by MMPs (Giannelli et al. 1997; Udayakumar et al. 2003). LN-5 is mainly deposited in BM, promoting static adhesion and hemidesmosome formation (Giannelli and Antonaci 2001) and mediating proliferation, cell migration, and tissue hemostasis. Migrating epithelial cells also synthesize laminin-1, collagens IV and VII, fibronectin, and integrins (Larjava et al. 1993). The data on normal intestinal wound healing are sparse. In ischemic colitis-type wound healing, MMPs-1, -7, and -10 are expressed in the migrating epithelium. In a fetal ileal model of wound healing, MMPs-1 and -10 show a normal expression pattern, but MMP-7 is not expressed (Salmela et al. 2004)(Figure 4).

In previous studies on human epidermal wound healing, MMPs-1, -9, -10, and -26 have been found in migrating keratinocytes in normally healing skin wounds, while TIMPs-1 and -3 are present at the mRNA level in the epidermis (Vaalamo et al. 1996, 1999; Saarialho-Kere 1998; Rechardt et al. 2000; Mirastschijski et al. 2002; Ahokas et al. 2005). TIMP-1 mRNA is also found in fibroblasts, macrophages, and endothelial cells in cutaneous wound healing (Vaalamo et al. 1999). MMP-3 is expressed in keratinocytes adjacent to the ulcer, but not in migrating keratinocytes (Vaalamo et al. 1996). MMP-12 is expressed by occasional stromal macrophages in acute dermal wounds (Vaalamo et al. 1999)(Figure 5).

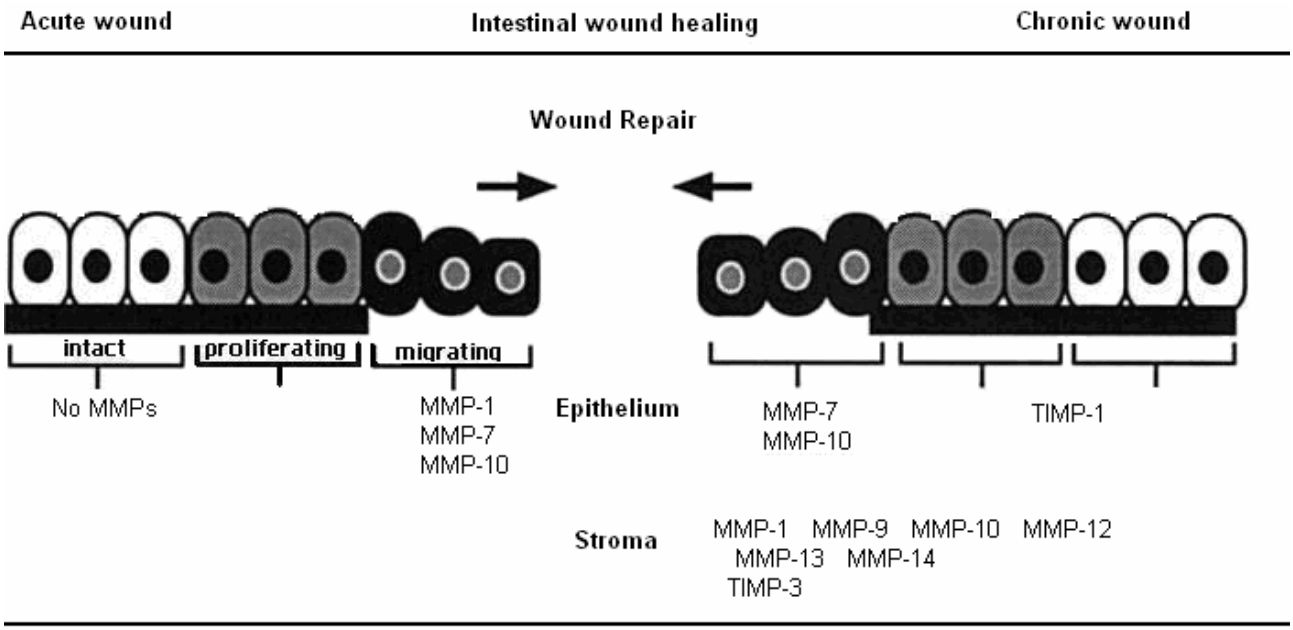

Figure 4. MMPs in intestinal wound healing (modified from Pilcher et al. 1998).

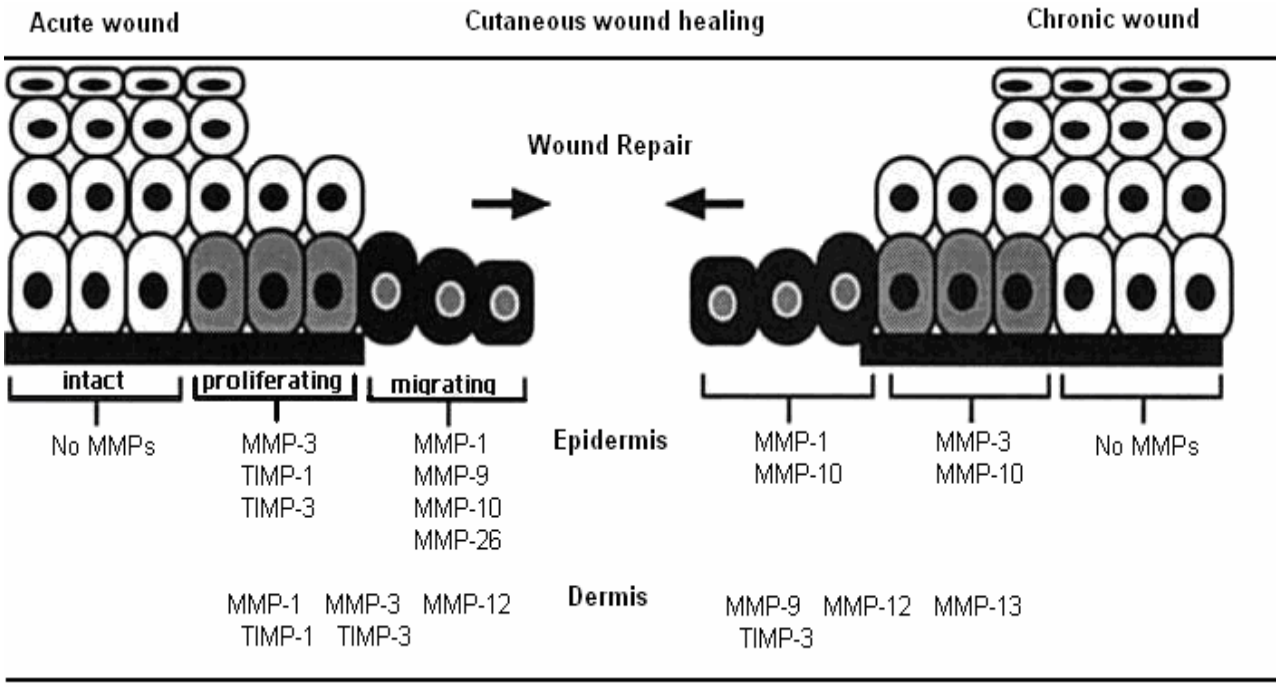

Figure 5. MMPs in cutaneous wound healing (modified from Pilcher et al. 1998 and Saarialho-Kere et al. 2002).

2.6.2 Delayed wound healing of the intestine and skin

In delayed wound healing, the normal repair process is impaired and disorganization of these four distinct phases occurs. Abundant inflammation and cytokine dysregulation characterize delayed wound healing. The delay may result from various causes. In the intestinal environment, the delay is usually caused by active inflammation and T-cell activation, as in IBD, or direct cytotoxicity by orally administered agents, as in CD. In cutaneous wound healing (Figures 3 and 5), the delay is usually caused by active inflammation, and hypoxia resulting from long-term hyperglycemia or pressure-induced wounds, as in diabetics, or sclerotic arterial structures, as in ASO patients, or general pressure in the lower extremities, as in patients with venous ulcers (Baker et al. 1992). The delay may also be due to bacterial infection, leading to excessive inflammation either in the intestine or in the skin (Falanga 2005). In chronic wounds, several kinds of white blood cells, i.e. macrophages, neutrophils, plasma cells, B-lymphocytes, T-lymphocytes, and mast cells, accumulate in the wound area (Senju et al. 1991; Loots et al. 1998; Huttunen et al. 2000). The amounts of different inflammatory cells vary depending on the wound etiology.

The epithelial repair activity is disturbed by various cytokines acting on improper matrix remodeling, formation of the new BM, and migrating potential of epithelial cells (Adair 1977; Grinnell et al. 1992; Falanga et al. 1994). Cytokines like IL-1, IL-6, and TNFα are upregulated in chronic cutaneous wounds (Trengove et al. 2000). Keratinocyte growth factor (KGF) improves mucosal healing in an experimental colitis model, and hepatocyte growth factor (HGF) and IL-6 enhance wound healing in IBD (Dignass et al. 1994; Zeeh et al. 1996; Tebbutt et al. 2002). In intestinal wound healing, MMPs-7 and -10 have been found in the migrating enterocytes expressing LN-5 while MMP-1 is absent (Saarialho-Kere et al. 1996; Vaalamo et al. 1998) (Figure 4). MMP-7 is upregulated in enterocytes by TNFα and IL-1β, bacterial exposure, and epithelial disruption (Lopez-Boado et al. 2000; Matsuno et al. 2003; Salmela et al. 2004). MMP-10 is upregulated by TNFα and EGF (Salmela et al. 2004). In delayed anastomotic healing, MMPs-1 and -2 are expressed in the mucosa and MMPs-2 and -9 in the submucosa (Stumpf et al. 2005). In MMP-3 KO mice, CD4 T-lymphocyte activity is diminished in the intestine, leading to impaired immunity to intestinal bacterial infection (Li CK et al. 2004). The amount of TIMP-1 mRNA in the intestinal epithelium increases according to the severity of the inflammation, i.e. ulcer formation (von Lampe et al. 2000). TIMP-1 is also upregulated by several isoforms of TGFβ in intestinal myofibroblasts, reducing stromal degradation (McKaig et al. 2003). TIMP-3 expression is very pronounced in fibroblast-like and endothelial cells in IBD stroma (Vaalamo et al. 1998). In another part of the GI tract, an experimental healing gastric ulcer expressed MMP-2 and TIMP-1 throughout all layers, but MMPs-9 and -13 only in the upper layers of the granulation tissue (Calabro et al. 2004).

In chronic cutaneous wounds, MMP-10 is expressed in migrating keratinocytes (Krampert et al. 2004), but MMPs-7 and -9 and TIMP-1 protein have not been found in keratinocytes (Vaalamo et al. 1999; Mirastschijski et al. 2002; Impola et al. 2005). Animal models have also demonstrated that MMP-10 is significantly increased in relation to controls during impaired cutaneous wound healing (Madlener et al. 1996) (Figure 5). MMP-9 expression is increased in diabetic foot ulcers compared with healthy controls´ traumatic wounds (Lobmann et al. 2002), as seen also in venous, decubitus, and rheumatoid ulcers. In MMP-9 KO mice, re-epithelization and inflammatory response are enhanced, but remodeling of the BM zone is impaired and an excessive amount of fibrin is deposited in the wounded

skin, cornea, or tracheal epithelium (Mohan et al. 2002). MMP-12 is expressed by stromal macrophages in chronic dermal wounds (Vaalamo et al. 1999). TIMP-3 expression is very pronounced in fibroblast-like and endothelial cells of chronic wounds (Vaalamo et al. 1999). MMP-13 is also upregulated in fibroblasts in chronic cutaneous wounds (Vaalamo et al. 1997). MMP-13 KO mice skin wounds have been found to heal normally (Hartenstein et al. 2006), which leads to the suggestion that this protein, which better resembles human MMP-1 than MMP-13, induces inflammation rather than keratinocyte migration in mice. MMP-3 is expressed in keratinocytes adjacent to the ulcer, but not in migrating keratinocytes (Vaalamo et al. 1996). In MMP-3 KO mice, contraction of cutaneous wounds is impaired, leading to retarded healing (Bullard et al. 1999).

2.6.3 Pyoderma gangrenosum

One of the most severe systemic defects affecting inflammatory reaction and the wound healing response is pyoderma gangrenosum (PG). This is a noninfectious reactive neutrophilic dermatosis that typically starts with folliculitis-like pustules and rapidly leads to painful ulcers of variable size and depth with surrounding violaceous borders. New ulcerations can often be induced even by minor trauma, a scratch, or a sharp needle, etc. PG was originally described in 1930 (Brunsting et al. 1930), but its pathogenesis remains unsolved, although an ever-widening range of systemic diseases has been described in association with it. Around two-thirds of PG cases are accompanied by an underlying disease, e.g. IBD, rheumatoid arthritis, or malignant hematological disease (Powell et al. 1985; Ho et al. 1992; Duguid et al. 1993). IBD is the most commonly associated systemic disease, underlying 10-50% of all PG cases (Sanders et al. 2001), although only 0.5-2% of IBD patients suffer from PG (Rothfuss et al. 2006). The diagnosis of PG is based on clinical and pathologic features and requires exclusion of other conditions that produce chronic ulcerations (i.e. infection, malignancy, rheumatologic disease). Histologically, tissue neutrophilia with epithelial undermining, perivascular and intramural lymphocytic infiltrates, and ulceration in the absence of leukocytic vasculitis characterize PG (Crowson et al. 2003). PG has five distinctive clinical and histologic variants (ulcerative, pustular, vesicopustular, bullous, and vegetative), and the specific clinical features of the lesion may provide a clue to the associated disease (Crowson et al. 2003). In general, treating a PG ulcer by traditional means, such as compression bandage, antibiotics, and local treatments, does not start the healing process; effective treatment is only achieved by systemic immunosuppression. To date, no single successful therapy exists for PG, but the treatments with the best clinical evidence are high-dose systemic corticosteroids and cyclosporine (Reichrath et al. 2005). In mild cases, also a combination of steroids and dapsone has been useful (Galun et al. 1986). Prevention of PG is achieved by treating the underlying disease. Despite advances in therapy, the long-term outcome for patients with PG remains unpredictable, with relapses being common (Conrad and Trueb 2005).

3. Aims of the study

Matrix metalloproteinases (MMPs) are a family of matrix-degrading enzymes. Classical MMPs have been found to be important in, for example, wound healing, arthritis, and tumor invasion. The recently cloned MMPs -19, -21, -26, and -28 and TIMP-4 have been our focus in this study. MMP-21 was first cloned by our research group in 2002. Matrilysin-1 (MMP-7) is found in migrating enterocytes in healing gut wounds and is connected to poor prognosis in cancers. Matrilysin-2 (MMP-26) is both structurally and functionally closely related to MMP-7, and TIMP-4 is its potent inhibitor. Therefore, the functions of MMP-26 and TIMP-4 are worth investigating in an intestinal context. MMP-28 is an epithelial MMP of the skin, the functions of which remain to be unraveled. Information on the specific tissues and the circumstances under which a proteinase is expressed are crucial to determine its function and a key event preceding the design of targeted inhibitors. MMPs can also be prognostic markers in cancers or other pathologic conditions.

Specific aims were the following:

I) To investigate the expression patterns and functions of the recently cloned MMPs-19, -26, and -28 in intestinal inflammation and colon cancer in adult patients.

II) To study the expression profile of several MMPs in the pathogenesis of necrotizing enterocolitis (NEC), an inflammatory condition affecting the neonatal intestine.

III) To investigate MMPs as putative markers of developing celiac disease in children and adolescents.

IV) To study the expression profile of MMPs in pyoderma gangrenosum, an example of a nonhealing wound associated with inflammatory bowel disease (IBD), and compare it with findings in IBD and normally healing wounds.

V) To investigate the roles of MMP-21, MMP-26, and TIMP-4 in pancreatic adenocarcinoma at a tissue level and their regulation in cultured pancreatic carcinoma cells.

4. Materials and methods

4.1 Tissue samples

These studies were approved by the Ethics Committees of the Department of Surgery (I), the Hospital for Children and Adolescents (II,III), and the Department of Medicine (IV,V), University of Helsinki, Helsinki University Central Hospital. All tissue samples were formalin-fixed and paraffin-embedded.

Gastrointestinal ulcers in adults (I): Peroperative specimens of ulcerative colitis (n=16) were obtained from the Department of Surgery, Helsinki University Central Hospital. Inflamed colonic samples were taken from two areas: those that macroscopically appeared normal and those demonstrating the highest degree of inflammation. Archival specimens of ischemic colitis (n=9), Crohn`s disease (n=7), ulcerative colitis (n=8), and healthy intestine (n=5) were obtained from the Department of Pathology, University of Helsinki, Finland.

Gastrointestinal inflammation in neonates (II): Peroperative specimens of necrotizing enterocolitis (NEC) (n=18, average gestational age 26.0 weeks, range 23.3–29.7, average age at operation 1.6 weeks, range 0–5.2) and necrosis (n=4, average gestational age 30.0 weeks, range 25.0–41.7, average age at operation 3.0 weeks, range 0.5–7.8), removed for clinical reasons and for routine histopathology, were obtained from the Departments of Pathology and Pediatrics, Helsinki University Central Hospital, Finland. Specimens of atresia (n=6, average gestational age 38.9 weeks, range 34.6–42.3, average age at operation 0.3 weeks, range 0–0.5) and the corresponding stomas (n=7, average gestational age 25.5 weeks, range 23.3–29.3, average age at operation 2.1 weeks, range 1.0–5.2) of NEC patients were studied as controls.

Celiac disease (CD) (III): Samples from 28 children (14 females, 14 males; median age 5 years, range 1.6-15) with undiagnostic histopathological findings in biopsies (n=14 with Marsh grade 0, n=10 with Marsh grade I (increase in intraepithelial lymphocytes), n=4 with Marsh grade II (cryptal hypertrophy)) and elevated IgA antibodies were included in the study. The samples were obtained from the Hospital for Children and Adolescents, Helsinki, Finland. A gluten-free diet was introduced immediately after the biopsy in eight cases by clinicians, based on the routine pathology report indicating changes suggestive of CD. Ten children were scheduled for a control visit 2-3 years after the biopsy, the other children attended a routine follow-up by pediatricians. Eight children were rebiopsied during the follow-up at clinicians´ request, and CD was diagnosed in two of them. Specimens of intestinal biopsies of patients with gastroesophageal reflux (GER) symptoms (n=6, 2 females, 4 males; median age 10 years, range 3-16) were used as controls.

Colon cancer (I): Specimens of colon cancer (n=20; Dukes grade A (n=5), grade B (n=7), grade C (n=4), and grade D (n=4)) were obtained from the Department of Pathology, University of Helsinki, Finland.

Pancreatic cancer (V): Archival specimens of pancreatic adenocarcinoma (n=25), carcinoma mucinosum (n=3), cystadenocarcinoma (n=2), malignant insulinoma (n=2), and benign pancreatic tumors (n=4) from 35 patients (18 males and 17 females, mean age 62 years, range 21-75), obtained during surgical removal in Whipple operations, were used.

In addition, 18 specimens representing the healthy margins of pancreatic tumors taken from the same patients were studied; these included samples of adenocarcinoma (n=13), carcinoma mucinosum (n=1), cystadenocarcinoma (n=2), and benign pancreatic tumors (n=2).

Pyoderma gangrenosum (PG) (IV): Archival specimens of PG patients (n=22, 24 samples altogether, 12 females, 10 males, median age 52 years, range 13-82) with PG representing its various subtypes (bullous (n=2), pustular (n=3), vegetative (n=9), ulcerative (n=5), vesicopustular (n=4)) were obtained from the Department of Dermatology, University of Helsinki, Finland. The biopsies had been taken before initiation of systemic treatment for PG. Four timed (1, 2, 3, and 4 days old) normally healing wounds from the upper thigh were also studied (Vaalamo et al. 1996).

4.2 Immunohistochemistry

Immunohistochemistry was performed using a streptavidin-biotin-peroxidase complex technique (DakoCytomation, StreptABComplex/HRP Duet, Mouse/Rabbit, Glostrup, Denmark, Vector mouse, Vector rabbit and Zymed). Diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) were used as chromogenic substrates and sections were counterstained with Mayer´s hematoxylin. After deparaffinization and rehydration, samples were pretreated, if necessary, with 10 mg/ml trypsin or a 95°C water bath for 30 min in DakoCytomation citrate-buffer or microwaving. After pretreatment, 0.5% hydrogen peroxide was applied to the slides to block endogenous peroxidase activity. Samples were immunostained using polyclonal or monoclonal antibodies (Table 3) and incubated for 1-2 h at room temperature, 30 min to 2 h at 37°C, or overnight at 4°C. Negative controls included rabbit pre-immune serum or rabbit IgG.

Preparation of MMP-26 antibodies. Antibodies were raised in rabbits against the recombinant protein expressed in *Eschericia coli* (Uria and López-Otin 2000). The specificity of the antibodies was demonstrated by Western blotting using solubilized recombinant protein produced in *Eschericia coli*, as previously described (Uria and López-Otin 2000). Briefly, 200 ng of solubilized recombinant protein was diluted 1:1 in Laemmli reducing sample buffer (Bio-Rad, Hercules, CA, USA) containing 5% ß-mercaptoethanol. Denatured proteins were separated on a 10% polyacrylamide gel, and the gel was blotted onto a Hybond C-extra (Amersham) membrane using standard protocols. Immunized rabbit antiserum was used as the primary antibody. Peroxidase-conjugated anti-rabbit IgG diluted 1:10000 in 0.1% Tween-20 / phosphate-buffered saline (PBS) containing 2.5% nonfat milk was used as the secondary antibody. The protein bands were visualized by chemiluminescence according to standard protocols.

Specificity of anti-MMP-26 antisera. To analyze MMP-26 protein, polyclonal antibodies were raised in rabbits by immunizing them with recombinant proMMP26. Western blotting of recombinant fusion proteins consisting of GST and partial length MMP-26 produced in *Eschericia coli* demonstrated two immunoreactive bands with expected molecular weights of approximately 29 kDa and 19 kDa, corresponding to the inactive and mature forms, respectively. With preimmune serum, no MMP-26 bands were detected (data not shown).

Table 3. Antibodies used in immunohistochemistry.

Antibody	Catalogue number	Source	Dilution	Pretreatment	Study
CD3	MoAbs-anti-Leu-4	Becton-Dickinson	1:400	-	III
CD68	M814	Dako	1:300	trypsin	I
Cytokeratin CAM 5.2	349205	Becton-Dickinson	1:75	pepsin	II
Fibronectin	MS-426	NeoMarkers	1:500	trypsin	I
Ki-67	M7240	Dako	1:75	microwave	II,III
Laminin-5		Prof.Karl Tryggvason, Karolinska Institutet	1:700	trypsin	I,II,IV
Tenascin-C	MAB1927	Chemicon	1:2000	trypsin	I
TNFα	ab9579-100	Abcam Ltd	1:50	trypsin	IV
Type IV collagen	M785	Dako	1:50	trypsin	I
MMP-1	IM35L	Calbiochem	1:500	trypsin	IV
MMP-7	IM40L	Calbiochem	1:80	-	I,II,IV,V
MMP-8	IM38L	Calbiochem	1:20	trypsin	IV
MMP-9	PC 213	Oncogene	1:50-75	trypsin	II
MMP-10	NCL-MMP-10	Novocastra laboratories Ltd.	1:300	trypsin	IV
MMP-19	PC 374	Oncogene	1:50-70	-	I,II,III
MMP-21		Sigma Genosys, Rehovot, Israel	1:150	trypsin	V
MMP-26		Prof Carlos López-Otin	1:400	-	I
MMP-26		Prof Keiichi Isaka	1:100-200	+95 C water bath	II,III,IV,V
MMP-28		Dr. Jouko Lohi	1:900	-	I
TIMP-1	IM63	Calbiochem	1:100	+95 C water bath	IV
TIMP-3	IM43L	Calbiochem	1:400	+95 C water bath	IV
TIMP-4	RB-1542	NeoMarkers	1:700	+95 C water bath	V

4.3 In situ hybridization

In situ hybridization. The production and specificity of MMP-1 (Saarialho-Kere et al. 1996; Vaalamo et al. 1997), MMP-3 (Saarialho-Kere et al. 1996), MMP-7 (Saarialho-Kere et al. 1996), MMP-10 (Saarialho-Kere et al. 1994; Vaalamo et al. 1998), and MMP-12 (Vaalamo et al. 1998) probes have been described previously. After deparaffinization and rehydration, 5 µm sections were pretreated with proteinase K (1 mg/ml) and washed in 0.1 M triethanolamine containing 0.25% acetic anhydride. Sections were hybridized overnight at 50-55^{o}C with ^{35}S-labeled probes and washed thereafter under stringent conditions and treated with RNAase A to remove unhybridized probe. After 20-40 days of autoradiographic exposure, the photographic emulsion was developed and the slides were stained with hematoxylin and eosin.

Table 4. Details on in situ hybridization probes.

Probe	Length bp	Part of sequence	Genbank accession no:	Study
MMP-1	550	1-550	NM_002421	II,III
MMP-3	217	1584-1801	NM_002422 tai XM_006271	III
MMP-7	800	14-813	NM_002423	II
MMP-10	175	1568-1743	NM_002425	II
MMP-12	651	600-1250	NM_002426	II,III

4.4 TUNEL staining

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick end labeling (TUNEL) method. Apoptotic cells were identified using the TUNEL method (Surh and Sprent 1994). 5 µm sections were dewaxed, rehydrated in alcohol, and permeabilized by microwave-pretreatment with 10 mM citric acid (pH 6.0). Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol, and the slides were incubated with terminal transferase reaction buffer essentially as described by Taskinen et al. (2004), with the exception that the titer for the anti-digoxigenin antibody was 1:100. The sections of the patients were always processed in parallel with those of the controls, and a total of two sections per patient were analyzed.

4.5 Cell cultures

Chemicals and materials. Cell culture media and fetal bovine serum (FBS) were from Cambrex (East Rutherford, NJ, USA), and cytokines and chemicals from Sigma (St. Louis, MO, USA). Cell culture plasticware was from Falcon, Becton-Dickinson (Franklin Lakes, NJ, USA), and Lab-Tek chamber slides from Nunc (Naperville, IL, USA). Reagents for real-time quantitative PCR were purchased from Applied Biosystems (Warrington, UK).

Cell culture models. Panc-1 cells (poorly differentiated carcinoma cells) (Lieber et al. 1975, ATCC, CRL-1469) were cultured in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% FBS, 2 mM of L-glutamine, 100 U of penicillin, and 100 µg/ml streptomycin. BxPc-3 (moderately to poorly differentiated carcinoma cells) (Tan et al. 1986, ATCC, CRL-1687) and AsPC-1 (well to poorly differentiated carcinoma cells) (Chen et al. 1982, ATCC, CRL-1682) were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM of L-glutamine, 100 U of penicillin, and 100 µg/ml streptomycin. The medium was changed three times a week. Cells were seeded as they reached confluence. After trypsinization, cells were seeded at a dilution of 1:2-1:4.

Cytokines and growth factors. To study the regulation of gene expression, cells were plated on six-well plates and grown overnight. The cells were then depleted of serum overnight prior to stimulation with different concentrations of tumor necrosis factor alpha (TNFα), transforming growth factor beta-1 (TGFβ1), interferon gamma (IFNγ), and epidermal growth factor (EGF) (Table 5). Stimulation was allowed to proceed for 24 h, after which the cells were lysed and total RNA was extracted using RNeasy Mini-kit (Qiagen) according to the manufacturer´s instructions. Cells in fresh serum-free medium were used as controls. All treatments were carried out in triplicate, and the results were confirmed in at least two independent experiments on all three cell lines. All cells were also grown on Lab-Tek chamber slides, and fixed with formalin for immunostaining 24 h after initiation of the culture (Ahokas et al. 2005).

Table 5. Cytokines used in this study.

Cytokine/Growth factor	Source	Concentration
TNFα	Sigma, St Louis, MO, USA	10ng/ml and 50ng/ml
TGFβ1	Sigma, St Louis, MO, USA	5ng/ml and 20ng/ml
IFNγ	Sigma, St Louis, MO, USA	10ng/ml and 25ng/ml
EGF	Sigma, St Louis, MO, USA	10ng/ml and 20ng/ml

4.6 Quantitative real-time PCR

Half a microgram of total cellular RNA from cultured cells was reverse-transcribed to cDNA with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) with oligo dTs and used as a template for TaqMan™ real-time quantitative PCR. Reactions were performed with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). PCR amplifications were performed in 10 µl volumes, MMP-21, MMP-26 or TIMP-4 and GAPDH in separate reactions. Primers and probes for MMP-21 and MMP-26 were as previously described (Ahokas et al. 2003, 2005), and TIMP-4 (Applied Biosystems: primer set Hs00162784_m1) and GAPDH (Applied Biosystems, VIC-TAMRA, Art. No. 4310884 E) assays were used. The mix contained 200 nM primers and a 100 nM probe for MMP-21 and MMP-26 or 0.5 µl of the 20× ready-to-use primer and probe mix for GAPDH and TIMP-4, 2 µl of the undiluted cDNA, and 1× Taqman™ Universal Master Mix. The PCR was started with 2 min at 50°C and an initial 10 min denaturation at 95°C, followed by a total of 40 cycles of 15 s denaturation at 95°C, and 1 min of annealing and elongation at 60°C. Human GAPDH labeled with VIC™ reporter dye (Pre-developed TaqMan™ assay reagents for endogenous control human GAPDH, Applied Biosystems) was used as an endogenous control in quantitative real-time PCR (TaqMan™). As a positive control for the expression of MMP-21, MMP-26, and TIMP-4 mRNA in conventional RT-PCR and Taqman analyses, we used placental mRNA as previously described (Ahokas et al. 2003, 2005).

4.7 Conventional PCR

Conventional PCR analyses were performed using the complementary DNAs (cDNAs) reverse-transcribed for real-time PCR analyses. Placenta poly(A) (Ambion, Austin, TX, USA) was used as a positive control for MMP-21, MMP-26, and TIMP-4 expression. PCR assays were performed in 25 µl volumes using 3 µl of cDNA for MMP-21 and TIMP-4/glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and 5 µl of cDNA for MMP-26/GAPDH, 200 nM of each primer [T21F, T21R, T26F, T26R] and 1.25 µl of TIMP-4 assay and GAPDH assay, 1× reaction buffer for DNA polymerase, 200 µM of each nucleotide, and 0.75 U of Ampli Taq Gold™ polymerase (Applied Biosystems). The PCR was started with 2 min at 50°C and an initial 10 min denaturation at 95°C, followed by a total of 30 cycles (MMP-21 and TIMP-4), 40 cycles (MMP-26), or 25 cycles (GAPDH) of 15 s denaturation at 95°C, and 1 min of annealing and elongation at 60°C. MMP-21, MMP-26, TIMP-4, and GAPDH reactions were done in separate tubes. The reaction products were run in a 2% agarose gel, stained with 5 ng/ml ethidium bromide, and visualized under ultraviolet light.

4.8 Statistical methods

Statistical analysis. Paired and unpaired t-tests were performed and a p-value under 0.05 was accepted as a significant result (III,IV,V). When antibody titers and cell numbers correlated with MMP findings, Spearman's rank correlation test or Mann-Whitney U-test was performed (III).

5. Results and Discussion

5.1 MMPs-19 and -28 are not associated with enterocyte migration in the inflamed intestine, unlike MMP-26 (I)

In the normal ileum, *MMP-19* protein was expressed in the apical areas of villi, with the intensity diminishing towards the villus base. In normal colonic surface epithelium, MMP-19 was found in the crypts. MMP-19 was upregulated by inflammation and could then be detected in fibroblasts, macrophages, and shedding epithelium in IBD, which is in accordance with previous data on cell types capable of expressing MMP-19 (Mauch et al. 2002; Hieta et al. 2003). MMP-19 was not detected in ulcerated areas in migrating enterocytes, as assessed by LN-5 staining on adjacent sections, but farther away from the ulcers. Consistent with observations in skin (Impola et al. 2003), MMP-19 protein was detected in areas where type IV collagen was absent. A cytokine highly relevant in IBD and other inflammatory conditions, TNFα, is able to induce MMP-19 expression in both keratinocytes and fibroblasts at least in vitro (Hieta et al. 2003; Impola et al. 2003). Like in cutaneous wounds (Impola et al. 2003), in psoriasis (Suomela et al. 2003), and in normal cutaneous glandular structures (Sadowski et al. 2003), MMP-19 was detected in the intestine in areas with proliferating cells such as colonic crypts. Thus, MMP-19 is a putative marker of epithelial cell proliferation in the intestine.

In the healthy ileum and colon, *MMP-26* protein was seen in a linear pattern underneath the epithelium in the BM zone. MMP-26 was detected in areas of intact epithelium, but the staining became blurred in areas of inflammation and shedding. In shedding epithelium, MMP-26 and its close relative, MMP-7, were found adjacent to each other: MMP-26 in the BM area and MMP-7 cytoplasmically in the shedding epithelium on top. MMP-26 was not expressed around the crypts, but in inflamed gut samples a reticulated pattern of staining was found surrounding the lowest crypts. MMP-26 was not seen in the cytoplasm of epithelial cells, except when the cells were migrating. This agrees with findings in cutaneous wounds (Ahokas et al. 2005) and in a human mucosal keratinocytes (HMK) wound model (Pirilä et al. 2007). However, this was not a constant phenomenon and was not observed in Crohn's disease with migrating cells. This may be due to different cell-matrix interactions capable of upregulating MMPs in these two disorders that display gut ulcers of different depths. The same kind of BM-associated, reticular immunostaining is also seen in acute and especially chronic cutaneous wound healing (Pirilä et al. 2007). MMP-26 is expressed in the early phase of acute wound repair and then disappears. In chronic wounds, MMP-26 is strongly expressed. However, discrepant results have also been reported (Ahokas et al. 2005), possibly due to different antibodies used in the studies. Upregulated MMP-26 expression is also seen in conjunction with inflammation in esophageal cancer (Ahokas et al. 2006). This would suggest that MMP-26 has an effect on epithelial cell migration as well as some kind of connection to inflammation. In addition, MMP-26 could be normally secreted by enterocytes and bound to some matrix components in the same manner as MMP-7 (Yu and Woessner 2000) or cleave integrins like MMP-7, which degrades β4 integrin (von Bredow et al. 1997). The synthetic MMP inhibitor ONO-4847 promoted wound repair in a dextran sulphate sodium (DSS)-induced colitis model in mice, possibly because it left MMP-1 and MMP-7, both important for enterocyte migration, uninhibited (Naito et al. 2004). Theoretically, these types of inhibitors could also be beneficial in human colitis.

MMP-28 was found in the apical regions of the villi in the normal ileum. Abundant expression was detected throughout the differentiated surface epithelium of the normal colon. In addition, the apical surface of the epithelial cells, the brush border, was occasionally positive. MMP-28 was not upregulated in migrating enterocytes, nor was it detected in any stromal cells in IBD or ischemic colitis. This is in agreement with data on cutaneous wounds, in which MMP-28 is not detected in migrating keratinocytes, but is found in proliferating keratinocytes, and is upregulated by TNFα (Saarialho-Kere et al. 2002). Inflammation did not seem to upregulate its expression. Unlike MMP-19, which was also expressed by macrophages and fibroblasts, MMP-28 was confined to epithelial structures. MMP-28 mRNA has also been detected in the mouse GI tract (Illman et al. 2003). MMP-28 mRNA is found in circulating T lymphocytes (Bar-Or et al. 2003), and protein in keratinocytes in hypertrophic scars (Reno et al. 2005). Based on our findings, MMP-28 may be involved in normal tissue turnover in the intestine.

5.2 MMPs-19 and -28 are downregulated in colon cancer, but MMP-26 is secreted into the nearby extracellular matrix (I)

Unlike many previously characterized MMPs, *MMP-19* was not present at the invasive front of colon cancers and was clearly downregulated in the epithelium of cancer cell islands. It could only be detected in occasional cancer cells and fibroblasts. Downregulation of MMP-19 in invasive cancer islands is in agreement with data on human breast and skin cancers (Djonov et al. 2001; Impola et al. 2003), in which MMP-19 is expressed by untransformed epithelial cells, but not when cells become invasive. Since MMP-19 has been found in low-grade cancers, it is suggested to be a protective marker. Recent studies have shown that MMP-19 cleaves nidogen-1 which is thought to stabilize microvessels (Titz et al. 2004). MMP-19-deficient mice transplanted with murine malignant keratinocytes show increased tumor invasion (Jost et al. 2006). Hence, MMP-19 is anti-angiogenic and probably slows down cancer progression. In contrast, in well-differentiated SCCs MMP-19 was found at the invasive front colocalizing with the migration marker LN-5 (Sadowski et al. 2005). In another MMP-19 KO mouse study, skin cancer susceptibility was reduced (Pendas et al. 2004). These results disagree with previous studies, but raise the question of whether MMP-19 is regulated differently in cancers of various origins.

In colon carcinomas, *MMP-26* was located neither in the BM area nor in the epithelia, but around the cancer cell islets in the matrix as a thread-like meshwork. Immunostaining for type IV collagen, fibronectin, and tenascin-C was therefore performed, but MMP-26 did not generally colocalize with them. It appeared to be downregulated, but not completely, in the areas of histologically more aggressive and dedifferentiated cancer, which agrees with results of other studies (Pilka et al. 2004; Lee et al. 2006). The expression pattern of MMP-26 in colon cancer suggests that cancer cells might have originally secreted this protein to the surrounding stroma. This finding would be interesting to confirm by immunostaining with another MMP-26 antibody (Isaka et al. 2003). BM staining may be associated with BM degradation and invasive progression, as seen in esophageal cancer (Ahokas et al. 2006). MMP-26 can activate proMMP-9, and both of these can degrade the BM (Zhao et al. 2004). In our study, staining was present around the cancer islets, but invasive progress was not detected in such areas. MMP-26 is also downregulated in endometrial carcinoma compared with in normal endometrial tissue (Isaka et al. 2003) and suggested to be important in reproductive events and implantation (Zhang et al. 2002; Li et al. 2002). MMP-

26 is found in several cancers (Pilka et al. 2004; Ripley et al. 2006; Lee 2006) and has been thought to be a protective marker because its expression decreases along with cellular dedifferentiation. Considering all data, however, evidence for this conclusion is somewhat contradictory. MMP-26 seems to be involved in cancers with high invasive potential, like its close relative MMP-7 (Zucker and Vacirca 2004), although it is not itself in the frontline all the time (Yamamoto et al. 2004; Ahokas et al. 2006). It could instead be involved in the transition phase of cellular dedifferentiation and tumor invasion. These results suggest that MMP-26 is a marker for poor prognosis in cancer staging.

In colon carcinomas, the surface epithelium was positive, but most of the colon cancers studied showed downregulation of *MMP-28* in the cancer islets. This is in accordance with data on commercial tumor tissue blots demonstrating downregulation of MMP-28 in colorectal cancer compared with in adjacent normal mucosa (Lohi and Illman, unpublished data). MMP-28 was absent from invasive colon cancer epithelium; the same kind of downregulation is also seen in skin cancer (Saarialho-Kere et al. 2002). On the other hand, MMP-28 has been shown to be upregulated in breast cancer, urothelial cancer, and oral squamous cell carcinoma, correlating with tumor grading (Overall et al. 2004; Illman et al. 2006; Lin et al. 2006; Wallard et al. 2006). MMP-28 has been suggested to enhance cancer invasion by activating other MMPs and by causing epithelial mesenchymal transition.

To find selective MMP inhibitors for curative purposes, clinical trials should be carried out in accurately chosen cancer types with punctual tumor grading. MMPs-19 and -28 were downregulated in the epithelium of invasive colon cancer islands in this study, apparently acting as “protective factors”, in contrast to the behavior of many classical MMPs involved in gastrointestinal cancer such as MMPs-1, -2, -7, -9, and -14 or TIMP-1 (Vihinen and Kähäri 2002; Turpeenniemi-Hujanen 2005). But, as observed in other cancer types, these results cannot be generalized. Furthermore, a higher number of colorectal cancer samples should be investigated in a larger study before drawing further conclusions. In this study (I), we concentrated mainly on MMP expression in response to intestinal inflammation, and as no data existed on these novel MMPs in cancer at that time, descriptive results in a small subset of colon cancers were reported.

5.3 Expression of MMPs-1, -7, -9, -12, and -26 is induced in necrotizing enterocolitis (II)

NEC is a major cause of morbidity and mortality in preterm infants. Together, prematurity, enteral feeding, and bacterial colonization have been hypothesized to result in an exaggerated inflammatory response, leading to ischemic bowel necrosis (Claud and Walker 2001). In this study, our major focus was on the putative contribution of a wide range of novel MMPs to NEC-associated tissue changes. Since MMPs probably act in a cascade-like fashion, as their function is regulated by activation by plasmin, furin, or other MMPs, and by TIMPs (Gomez et al. 1997), it is important to investigate the contribution of several MMPs to NEC simultaneously.

MMP-1 mRNA was expressed in 8/18 NEC samples in mesenchymal fibroblast-like cells, correlating with the presence of acute necrosis. MMP-1 was also detected in the epithelium of 5/18 NEC samples and three stomas in regenerative areas. Moreover, 2/7 control stomas, 2/6 samples of atresia, and 2/4 samples of necrosis were also positive for

stromal MMP-1 mRNA. A number of studies suggest that MMPs are the most important group of proteolytic enzymes responsible for the breakdown of ECM in another disorder characterized by intestinal tissue destruction, namely UC (Vaalamo et al. 1998; Baugh et al. 1999; Heuschkel et al. 2001; Salmela et al. 2002; Matsuno et al. 2003). MMP-1 was detected in half of our NEC samples in fibroblast-like cells of the stroma. In IBD, MMP-1 is involved in mucosal destruction (Stallmach et al. 2000; Di Sebastiano et al. 2001) and our stromal MMP-1 expression may well reflect tissue injury in NEC. MMP-1 was also detected in the epithelial cells of regenerating areas, unlike in IBD (Saarialho-Kere et al. 1996; Arihiro et al. 2001; Matsuno et al. 2003), and this agrees with previous results on ischemic colitis (Salmela et al. 2004), which resembles NEC more than IBD.

MMP-7 expression was most intensive in the injured epithelium of 12/17 NEC samples, but the intensity decreased towards the necrotic areas. MMP-7 was also detected in the regenerating mucosal epithelium in both NEC and necrosis specimens. Moreover, positive epithelial cells were found in atresia samples (2/6), in necrotic samples (3/4), and in control stomas (3/7). However, the number of positive cells did not equal that of NEC samples. MMP-7 is strongly expressed in NEC, but not generally in control stomas, suggesting that it is of significance in tissue destruction in NEC. Expression of MMP-7 has been discovered to correlate with the degree of inflammation in UC (Matsuno et al. 2003). In the small intestine, MMP-7 functions in host defense by activating defensins (Parks et al. 2001). Bacterial exposure is a potent signal regulating MMP-7 expression in epithelial cells (Lopez-Boado et al. 2000) and may also be the mechanism for its induction in NEC since the disease onset usually correlates with the beginning of oral feeding. Epithelial disruption is needed for MMP-7 induction in human intestinal epithelial cells in vivo (Saarialho-Kere et al. 1996; Matsuno et al. 2003), as seen in IBD (Rath et al. 2006). MMP-7 is also found in precancerous polyps in colonic mucosa, indicating that it may have a role in early carcinogenesis (Rath et al. 2006).

MMP-9 expression was detected in neutrophils in the majority of NEC, stoma, and atresia samples. MMP-9-positive macrophages were also detected in a subpopulation of all diagnostic categories examined in the areas of serosa and neutrophil accumulations. Control stomas had the same expression of MMP-9 as the original NEC sample from the same patient, but the positive staining concentrated also in the submucosa along the cutting edge of the stoma. MMP-9 has been shown to be a major factor in adult intestinal tissue destruction and inflammation; e.g. in IBD patients, colitis models, ischemic colitis patients (Baugh et al. 1999; Tarlton et al. 2000; Medina et al. 2006), and recently in MMP-9 KO mice with significantly reduced extent and severity of colitis when exposed to dextran sodium sulfate or Salmonella typhimurium (Castaneda et al. 2005). An experimental obstruction model in rats demonstrated that colonic obstruction and trauma upregulate gelatinases and decrease collagen concentration in the colonic wall (Syk et al. 2003). Hence, obstruction and local trauma may be important in upregulating leukocyte MMP-9 activity also in atresia samples.

MMP-12 was expressed by macrophages inside the villi and in the stroma underlying the crypts in 7/18 NEC specimens. Of the stomas, 2/7 were positive. Overall expression in all NEC samples was more intense than in the positive stomas. Atresia samples were negative for MMP-12, reflecting the absence of widespread inflammation in these samples, whereas 2/4 samples with necrosis were positive. MMP-12 has recently been found to destroy epithelial cells in cultured intestinal cells. Additionally, when trinitrobenzene sulfonic acid (TNBS) was administered to the colons of MMP-12 KO mice for 1 week, no

mucosal damage was seen histologically compared with wild-type controls with severe colitis. Colons of wild-type mice, but not of KO mice, were significantly thickened after TNBS treatment (Pender et al. 2006). This is in line with our results and confirms the importance of MMP-12 in colitis-induced epithelial destruction.

MMP-26 was expressed in the fairly intact villous epithelium of the ileum. In NEC samples, its expression was downregulated in the epithelium of injured, inflamed areas, but general expression was seen in 12/17 samples. Stromal cells were positive for MMP-26 in NEC, especially macrophages in the regenerative areas. Also the vascular endothelium showed positive staining in several samples. In stoma samples, the mucosal epithelium was generally negative and stromal signal was only occasionally detected, unlike in NEC samples. MMP-26 is involved in enterocyte and keratinocyte migration over ulcerated areas (Ahokas et al. 2005). In NEC, we detected MMP-26 also in endothelial cells, agreeing with a recent report on endometrial cancer (Tunuguntla et al. 2003). Upregulation of MMP-26 in the stromal macrophages of NEC samples was marked. This macrophage-associated staining has been confirmed by Li W et al. (2004), who found MMP-26 not only in macrophages but also in neutrophils. Skoog et al. (2006) recently reported MMP-26 expression in macrophages of cutaneous granulomas as well as in two cultured fibroblast cell lines.

MMP-10 was expressed by fibroblast-like cells in the stroma around the crypts in 3/18 NEC specimens only. Expression was found in mesenchymal cells in acute necrosis and regenerative areas. Of the stomas, 4/7 were positive for MMP-10. MMP–10 is a potent activator of proMMP-1. It was rarely noted in stromal cells in NEC and was absent from epithelial cells. In an experimental model of IBD, MMP-10 was expressed in areas with the most severe injury (Salmela et al. 2002), and stromal expression in NEC probably reflects the same phenomenon.

Only 5/18 NEC samples had positive immunosignal for *MMP-19*. MMP-19 protein was detected in the crypt epithelium. All stoma samples displayed epithelial expression of MMP-19. The overall expression in stoma samples was more intense and even than in NEC samples. MMP-19 has been found in fibroblasts, myoepithelial, and smooth muscle cells as well as in association with the cell surface of myeloid cells (Kolb et al. 1999; Mauch et al. 2002). The absence of MMP-19 in the majority of NEC samples with severe tissue injury may reflect its role in normal tissue turnover (Kolb et al. 1999). Our research group has detected MMP-19 in hyperproliferating keratinocytes (Impola et al. 2003; Suomela et al. 2003), and thus, its presence particularly in the cryptal epithelium of stomas may be associated with enterocyte proliferation.

While our study was in progress, Pender et al. (2003) reported increased expression for MMP-3 in myofibroblasts of NEC patients in vivo, suggesting that MMP-3, but not MMP-1 or MMP–9, is responsible for the extensive tissue injury seen in NEC. Our study extends and partly confirms their findings; the differences observed may be explained by our control and NEC samples representing patients of similar ages, while the ages of NEC patients and controls were significantly different (10 days vs. 4-5 months) in the study of Pender et al. (2003).

Increased levels of TNFα and decreased levels of IFNγ have been noted in vivo in human NEC (Pender et al. 2003). In experimental models, TNFα is known to cause tissue injury by stimulating mucosal mesenchymal cells to secrete MMPs, and TNFα antibodies can

block this cascade (Pender et al. 1998). TNFα can upregulate several MMPs, such as MMP-9 and MMP-12, in macrophages (Saren et al. 1996; Churg et al. 2003), MMP-19 in epithelial cells (Impola et al. 2003), and MMPs-1, –7, and -10 in colon carcinoma (Caco-2, WiDR, and HT-29) cell cultures (Salmela et al. 2004). TNFα exposure to myofibroblasts induces MMP-1 and -3 secretion (Okuno et al. 2002; Bamba et al. 2003). TNFα also downregulates TIMP-1 expression (Yao et al. 1997), which favors matrix breakdown. Therefore, TNFα may well be the crucial cytokine upregulating MMPs and thereby inducing tissue destruction in NEC.

5.4 MMP-12 is a putative marker for latent celiac disease (III)

MMP-12 was detected in large, plump, macrophage-like cells in 16/28 gut biopsies obtained from children. Two children with confirmed CD during follow-up and all children on a gluten-free diet on clinicians´ decision, had MMP-12 mRNA-positive cells in their samples. Among the other children with sustained elevation of TG2 antibodies (n=6), two children had a moderately positive signal for MMP-12 mRNA and the remaining four were negative. Their re-biopsies did not fulfill the criteria for CD. Occasional MMP-12-positive cells were detected in 3/12 biopsies of the children with normalized antibodies, but most of the biopsies from children whose antibodies normalized during follow-up were negative for MMP-12. Overall, expression of MMP-12-positive cells in patients with CD or without CD was 1.56 and 0.5, respectively ($p<0.001$). Upregulation of MMP-12 expression was associated with increased titers for TG2 and EMA antibodies, increased numbers of CD3 and gamma/delta cells, and a high percentage of cryptal Ki-67-positive cells. Tunel villus cell counts did not correlate with MMP-12 levels.

In macrophages in culture, TNFα is known to upregulate MMP-12 (Feinberg et al. 2000), and thus, the upregulation of this cytokine in potential CD could be responsible for the induction of MMP-12. In addition to the celiac intestine, MMP-12 is upregulated in experimental models of T-cell-mediated tissue injury of the intestine (Salmela et al. 2001, 2002), and animal studies suggest that MMP-12 may partly contribute to cryptal hyperplasia (Li CK et al. 2004). However, as in our control samples, MMP-12 cannot be detected in the normal adult jejunum or ileum (Salmela et al. 2001). It may help macrophages to migrate to sites of inflammation by degrading BMs of endothelial cells and the mucosal epithelium. MMP-12 may also regulate inflammatory response since it can activate TNFα (Chandler et al. 1996). In this study (III), the main finding was that the presence of MMP-12-positive macrophages correlates not only with the number of gamma/delta cells, but also with EMA and TG2 antibodies in pediatric patients with potential CD. In other studies, MMP-12 is also suggested to be of importance in the cutaneous manifestation of CD, dermatitis herpetiformis (Salmela et al. 2001), in severity of CD (Ciccocioppo et al. 2005), and in direct intestinal tissue destruction (Pender et al. 2006). Therefore, patients with potential CD with clinical symptoms and positive MMP-12 staining in the initial biopsy, but no clear villus atrophy, should udergo repeated follow-up.

5.5 MMP-26, but not MMPs-1, -3, and -19, are upregulated in latent celiac disease (III)

MMP-26 protein was detected in plump macrophage-like cells in 12/28 biopsies. The presence of these cells was associated with high antibody titers. Endothelial MMP-26 was detected in all patient samples. MMP-26 protein was detected in plump macrophage-like cells in 1/6 control samples and in endothelial cells in all of them. Although the frequency of MMP-26-positive samples seemed to increase with CD3 number and EMA and TG2 antibodies, their numbers in samples were not associated with the permanence of these antibodies and development of CD. An epithelial signal was not detected since ulcerations are needed for upregulation of MMP-26 in enterocytes. MMP-26 was detected in endothelial cells and macrophages, consistent with our NEC study. These results suggest that MMP-26 is involved in inflammation and tissue regeneration since it is found in macrophages at inflamed sites and in migrating enterocytes when ulcers are present.

MMPs-1 and -3, previously reported to be associated with overt CD (Daum et al. 1999; Ciccocioppo et al. 2005), were very occasionally expressed. MMP-1 mRNA was detected in stromal and epithelial cells in 4/26 samples. Only one of these MMP-1 mRNA-positive children had CD on clinicians´ decision (gluten-free diet), and MMP-1 expression did not correlate with EMA/TG2 antibodies. MMP-3 mRNA was detected in 2/28 samples, but neither of these patients had CD. MMP-1 or MMP-3 mRNA was not found in control samples. In IBD, MMP-1 is involved in mucosal destruction through degradation of several collagen types (Stallmach et al. 2000). In CD, MMP-1 could contribute to villous atrophy via degrading collagen types I and III, and MMP-3 to epithelial cell shedding (Daum et al. 1999). TIMP-1 is also elevated in celiac disease (Ciccocioppo et al. 2005), and it inhibits mainly MMP-1. Hence, the inflammation in potential CD is somewhat lower grade than in other inflammatory bowel conditions, and the TIMP/MMP ratio seems to be optimal for preventing tissue destruction. MMP-3 gene allele 6A has been implicated as a risk factor for men contracting CD (Mora et al. 2005).

MMP-19 was found in the epithelium of 15/28 samples. It was more often seen in children with low antibody titers than in those with CD or CD on clinicians´ decision (i.e. gluten-free diet). The number of stromal MMP-19-positive macrophages tended to increase with elevated CD3 cells and TG2 antibodies. Among children whose antibody levels normalized during follow-up, MMP-19-positive macrophages were an occasional finding. Epithelial immunostaining for MMP-19 was detected in 3/6 control samples. Epithelial staining was more often seen in controls and patients with normal titers than in those with CD, probably reflecting MMP-19 functioning in the normal turnover of the intestine. On the other hand, MMP-19 has also been suggested to be involved in macrophage migration through endothelial cells and the stromal compartment (Mauch 2003) since it is found to be a cell surface-associated protein (Mauch et al. 2003). Several other MMPs, including MMPs-9, -12, and -26, have been found in T cells (Leppert et al. 1995; Hughes et al. 1998; Li CK et al. 2004; Marchenko et al. 2004).

When the children with CD (n=10; including those with a gluten-free diet started by clinicians´ decision or CD confirmed in re-biopsy) were compared with those who did not develop CD (n=18), the numbers of IEL and cryptal proliferative cells were higher (IEL 0.9 and 0.28, $p<0.001$, and cryptal proliferative cells 1.88 and 1.06, $p<0.004$), as were the numbers of CD3-positive cells (75.6 and 36.6, $p<0.004$). A raised IEL count with normal villous architecture is a recognized finding in latent CD. Counting of IELs is recommended

in borderline cases where the histology is difficult to interpret; an increase in gamma/delta positive cells strengthens the probability of CD (Järvinen et al. 2003). The densities of IFNγ- and TNFα-positive cells are increased in potential CD patients with high IELs (Westerholm-Ormio et al. 2002). Furthermore, T cells and macrophages in the lamina propria are thought to play a central role in the pathogenesis of CD through their secretion of cytokines, which can be identified long before villous changes occur. Villous atrophy in CD has been ascribed to an IEL-mediated damage to enterocytes involving NKG2D/MICA on the intestinal epithelium (Hue et al. 2004). MIC transmembrane molecules can be shed after proteolytic cleavage, and thus, increased production of MMPs (Daum et al. 1999; Salmela et al. 2001) might favor the release of sMIC. A slight or moderate increase in TG2 antibodies together with low titers of EMA in patient serum, but inadequate criteria for diagnosis of CD in biopsy, poses a challenge for clinicians. In this study, we noted that the positive screening tests for these antibodies became negative during a 1- to 2-year follow-up in one-third of cases. A re-biopsy was performed in one-third of children (n=8) because their TG2 antibodies remained high or even increased during the follow-up. In these re-biopsies, only 2/8 patients met the morphologic criteria for CD. The remainder of the re-biopsied patients might also be diagnosed with CD after a longer follow-up period. Nevertheless, MMP-12 staining may be helpful in distinguishing patients with the highest risk for later onset of CD, but a larger patient cohort is needed to confirm this hypothesis.

5.6 MMPs-1 and -12 are expressed in the intestine of type 1 diabetes patients (III)

CD and T1D frequently co-exist due to a common genetic predisposition, namely HLA class II heterodimer HLA-DQ2 or HLA-DQ8 (Ilonen et al. 1996); the prevalence of CD in diabetic children in the Caucasian population has been estimated to vary between 1% and 10% (Holmes 2002). Therefore, children with T1D are routinely screened for the presence of CD-specific antibodies. We also studied the histological and MMP profile in the intestine of T1D patients. When patients with T1D (n=10) were compared with those who did not have diabetes, *MMP-12* and *MMP-1* mRNA-positive cells were detected more frequently in T1D patients. All children with a positive MMP-1 mRNA finding (n=4) had T1D and were also positive for MMP-12. Only 2/10 children with T1D were negative for MMP-12. The only two patients who had MMP-3-positive cells in their biopsies had T1D. There was no difference in MMP-19 and MMP-26 positivity when children with T1D were compared with those without T1D. The number of apoptotic cells in the mucosal surface epithelium tended to be higher in children with T1D than in the other children, but the results were not statistically significant. Furthermore, significant changes were not observed in EMA or TG2 titers, numbers of plasma cells, neutrophils, eosinophils, IELs or gamma/delta cells, or in numbers of Ki-67- and CD3-positive cells.

All MMP-1 and -3 mRNA-positive cases had T1D, and also MMP-12 was frequently detected in the diabetic gut. A stage of bowel inflammation has been reported in patients with T1D (Savilahti et al. 1999), although their gamma/delta cell numbers are not different from controls. This could partly explain our findings of MMPs-1, -3, and -12. A recent finding shows that densities of IL-1- and IL-4-positive cells are increased in T1D patients with normal villous structure, while IL-2-, IFNγ-, and TNFα-positive cells correlate with the presence of CD in patients with T1D (Westerholm-Ormio et al. 2003). Aberrant function of the gut immune system, such as intestinal immune activation and increased intestinal permeability, is characteristic of T1D, and thus, T cells may be sensitized to act

aggressively on wheat proteins (Savilahti et al. 1999; Westerholm-Ormio et al. 2003). TIMP-1 levels in blood samples are elevated in patients with T1D (Maxwell et al. 2001; Jacqueminet et al. 2006). TIMP-1 is a potent inhibitor of MMP-1, which strengthens the idea that MMP-1 might be involved in minor intestinal inflammation in T1D patients. This expression could precede the onset of CD. Furthermore, MMP-9 and TIMP-2 are elevated in circulating blood of patients with T1D, with or without microvascular complications, and MMP-2 is upregulated or sustained in T1D patients, but is suggested to be a marker of microangiopathy (Maxwell et al. 2001; Derosa et al. 2005, 2007; Jacqueminet et al. 2006; Shiau et al. 2006).

5.7 Lack of epithelial MMPs-1 and -26 characterizes retarded wound healing (IV)

PG is an extraintestinal, cutaneous manifestation of IBD. It is also a very good model of a chronic cutaneous wound. We therefore compared the MMP profiles of PG, IBD, and a normally healing cutaneous wound.

MMP-1. Epithelial expression for MMP-1 was detected in 21/24 PG samples. Migrating keratinocytes were positive in 6/24 and the epithelium farther away from the ulcer edge in 20/24 samples. Stromal expression for MMP-1 was seen in 20/24 samples in fibroblasts and macrophages. MMP-1 was diminished in migrating keratinocytes of 5- to 12-month-old wounds compared with wounds less than 4 months old (0 vs. 0.286, $p<0.04$). When PG lesions of patients with underlying IBD were compared with those of nonIBD patients, MMP-1 was diminished in cells with migratory potential (0 vs. 0.286, $p<0.04$) in IBD patients. In acute wounds, only the migrating epidermal tip and occasional stromal cells were positive for MMP-1 protein. Normal cutaneous re-epithelialization involves MMPs-1, -9, -10, and -26 in migrating keratinocytes (Kerkelä and Saarialho-Kere 2003; Ahokas et al. 2005). MMP-1 is instrumental for keratinocyte migration on type I collagen in healing wounds (Pilcher et al. 1998), but MMP-1 protein was absent in the migrating front of a majority of PG ulcers. This may partly explain the chronic unhealing nature of PG without immunosuppressive treatment. MMP-1 mRNA has been detected in the granulation tissue in IBD ulcers (Saarialho-Kere et al. 1996), in inflammatory cells and fibroblasts (Arihiro et al. 2001), as seen in the stroma of PG. In the gut, stromal MMP-1 has been associated with tissue destruction in experimental models (Pender et al. 1998). Furthermore, migrating enterocytes bordering intestinal ulcers express MMP-1 during regeneration, at least in ischemic colitis (Salmela et al. 2004). As in PG, stromal MMP-1 expression is strongly increased in chronic diabetic foot ulcers compared with traumatic wounds in healthy controls (Lobmann et al. 2002).

MMP-26. Epithelial expression of MMP-26 was positive in 10/24 samples. Migrating keratinocytes were positive in 10/24 samples, while MMP-26 was not detected in the epithelium farther away from the wound edge. Stromal expression was generally not detected. In acute wounds, MMP-26 was expressed by the migrating keratinocytes, as seen in a previous in vivo study in LN-5-positive migrating keratinocytes (Ahokas et al. 2005). In PG, migrating keratinocytes expressed MMP-26 more in patients who were treated with immunosuppressants (0.727 vs. 0.154, $p<0.004$), which also supports the beneficial role of MMP-26 in wound healing (Ahokas et al. 2005). MMP-26 can degrade several BM and ECM components (Park et al. 2000). MMP-26 was rarely expressed in the epithelium of PG, and its absence may contribute to retarded re-epithelization by impaired

BM remodeling, which was confirmed by a recent HMK migration study (Pirila et al. 2007), or MMP-9 activation (Zhao et al. 2003). MMP-26 was generally detected in patients with ongoing immunosuppressant treatment. However, at least in vitro corticosteroids cannot induce MMP-26 expression in keratinocytes (Ahokas et al. 2005). Estrogen is known to influence wound repair (Ashcroft and Ashworth 2003), and indeed, MMP-26 could be upregulated by it also in keratinocytes (Pilka et al. 2006).

5.8 MMPs-8, -9, and -10 and TNFα are upregulated in pyoderma gangrenosum (IV)

MMP-7. Epithelial expression of MMP-7 was found in 1/20 PG samples farther away from the ulcer edge. Stromal expression was found in 4/20 samples in macrophage-like cells. As previously shown in other types of chronic wounds (Impola et al. 2005), MMP-7 is not present in keratinocytes, and this agrees with our negative findings in PG. However, MMP-7 mRNA and protein are expressed in intestinal ulcerations by migrating enterocytes, and MMP-7 mRNA is upregulated by TNFα and IL-1β in enterocytes (Saarialho-Kere et al. 1996; Salmela et al. 2004). MMP-7 was detected only in occasional macrophages in four PG samples, and thus, unlike in the intestine where MMP-7 expression increases corresponding to the severity of inflammation (Matsuno et al. 2003), the degree of inflammation has no effect on MMP-7 expression in the skin. MMP-7 expression is also associated with intestinal bacterial exposure (Lopez-Boado et al. 2000), but no data exist on whether MMP-7 also processes antimicrobial peptides in cutaneous inflammation.

MMP-8 was not detected in the epithelium of any of the PG samples, but was observed in half of the neutrophils. In acute wounds, occasional stromal neutrophils were positive for MMP-8, but none were detected in keratinocytes. Absence of epidermal MMP-8 and high levels of stromal MMP-8 expression characterize both chronic venous ulcers and diabetic ulcers (Nwomeh et al. 1999; Lobmann et al. 2002; Impola et al. 2005), and this MMP may well contribute to stromal tissue destruction also in PG. In a recent MMP-8 KO mouse study, the degree of inflammation and expression of MMP-9 were increased, resulting in delayed wound repair in acute wounds (Gutierrez-Fernandez et al. 2007). The authors suggested that the excessive inflammation is a consequence of MMP-9 overexpression; a natural balance may exist between these two proteases or perhaps MMP-8 has the capability of deactivating MMP-9.

MMP-9. Epithelial expression of MMP-9 was detected in 8/24 PG samples. Migrating keratinocytes were positive in 7/24 samples and in the epithelium farther away from the ulcer edge in 2/24 samples. Stromal expression for MMP-9 was detected in 23/24 samples in macrophages and neutrophils. The most abundant staining for MMP-9 was generally detected in the ECM under the epithelium and in the wound bed. MMP-9 was diminished in the migrating keratinocytes of 5- to 12-month-old wounds compared with wounds under 4 months of age (0.286 vs. 0, $p<0.04$). When PG lesions of patients with underlying IBD were compared with those of nonIBD patients, stromal MMP-9 staining was increased (2.0 vs. 1.429, $p<0.01$) in IBD patients. In acute wounds, the migrating epithelium was positive for MMP-9, as were occasional stromal neutrophils and macrophages. MMP-9 is upregulated in migrating keratinocytes in vivo (Saarialho-Kere et al. 2002), and may participate in remodeling of the BM (Mirastschijski et al. 2002). Similar to venous, decubitus, and rheumatoid ulcers, MMP-9 expression is increased in diabetic foot ulcers compared with wounds of healthy controls (Lobmann et al. 2002). MMP-9 is reported to be

rarely expressed by keratinocytes in chronic cutaneous wounds (Mirastschijski et al. 2002), agreeing with the epithelial expression results of the present study. However, we found abundant ECM-associated MMP-9 expression in PG, suggesting that this MMP may essentially contribute to the poor healing capacity of this wound type, which correlates with results in MMP-9 KO mice (Mohan et al. 2002). MMP-9 is also an important contributor to intestinal tissue destruction and is expressed by inflammatory cells, fibroblastic cells, and vascular smooth muscle cells in the inflamed intestine (Leppert et al. 1998; Pender et al. 1998; Baugh et al. 1999) and in fistulae caused by Crohn´s disease (Kirkegaard et al. 2004). Increased expression of MMP-9 in the ECM of IBD lesions correlates with the severity of inflammation (Gao et al. 2005), which is also seen in PG. MMP-9 is upregulated by TNFα in a variety of cell types (Van den Steen et al. 2002), particularly during cutaneous wound repair (Scott et al. 2004). Thus, the TNFα abundantly present in PG may induce MMP-9 expression.

MMP-10. Epithelial expression for MMP-10 was detected in 21/24 PG samples. The migrating keratinocytes were positive in 11/24 samples, and the epithelium farther away from the ulcer edge in 20/24 samples. The ulcer margin overhanging the dermal neutrophilic infiltrate (the active zone of dermolysis) expressed MMP-10 intensively. Stromal expression of MMP-10 was seen in 18/24 samples in macrophages, fibroblasts, and endothelial cells. In acute wounds, migrating keratinocytes always expressed MMP-10, while only occasional stromal cells were positive. In keratinocytes, MMP-10 enhances cell migration and participates in the remodeling of LN-5 (Krampert et al. 2004). Although MMP-10 is detected in a limited number of cells in the migrating front of normally healing wounds (Rechardt et al. 2000), strong epithelial and also stromal expression was evident in PG. Abundant expression of MMP-10 in the epithelium may contribute to excessive degradation of collagen IV, fibronectin, and nidogen in the BM zone (Salmela et al. 2002) and inhibit the formation of new BM. MMP-10 is expressed by migrating enterocytes bordering intestinal ulcers in IBD, and it is upregulated by TNFα and EGF (Vaalamo et al. 1998; Salmela et al. 2004). It has been found in the stroma of a T-cell explant model of mucosal destruction (Salmela et al. 2002). Also animal models have demonstrated that MMP-10 is significantly increased in relation to controls during impaired wound healing (Madlener et al. 1996). Thus, strong stromal expression of MMP-10, unlike that seen in normally healing wounds (Rechardt et al. 2000), may contribute to retardation of healing in PG.

*TNF*α*.* Epithelial expression for TNFα was detected in 4/24 PG samples. The migrating keratinocytes were positive in 3/24 samples, and the epithelium farther away from the ulcer edge in 2/24 samples. Stromal expression of TNFα was positive in 23/24 samples in fibroblasts, macrophages, and neutrophils. In acute wounds, TNFα was expressed by migrating keratinocytes and occasional stromal neutrophils, macrophages, and fibroblasts. TNFα is a potent activator of several MMPs that contribute to ECM and BM degradation and overall tissue destruction (Gan et al. 2001; Nee et al. 2004), and it is also upregulated in psoriasis (Nickoloff et al. 2006). As shown here, TNFα expression is very abundant in PG. Based on these findings, TNFα inhibitors could offer a therapeutic option for PG. In fact, the TNFα inhibitor infliximab has already shown promising results in treating patients with PG (Brooklyn et al. 2006).

5.9 TIMP-1 expression is elevated in the stroma in response to inflammation, and epithelial TIMP-3 expression may retard wound healing (IV)

TIMP-1 was not found in the epithelium of any of the PG specimens. Positive stromal expression in macrophages, fibroblasts, and endothelial cells was detected in 19/24 samples. TIMP-1 protein was not expressed by keratinocytes in normally healing wounds, but only in fibroblasts of the stroma. Epidermal expression of TIMP-1 mRNA has not been found in chronic wounds. In acute wounds, expression has been detected in proliferating keratinocytes (Vaalamo et al. 1999). TIMP-1 has been detected in acute and chronic cutaneous wound healing stromally in fibroblasts, macrophages and endothelial cells (Vaalamo et al. 1999): the same cell types showed moderate expression in the stroma of most PGs. In the inflamed intestine, TIMP-1 is expressed by inflammatory cells, fibroblastic cells, and vascular smooth muscle cells (Saarialho-Kere et al. 1996; Vaalamo et al. 1998; Arihiro et al. 2001). The amount of TIMP-1 mRNA in the intestinal epithelium depends on the severity of the inflammation, namely ulcers (von Lampe et al. 2000).

TIMP-3. Epithelial expression of TIMP-3 was positive in all PG samples. The migrating keratinocytes were positive in 18/20 samples, and the epithelium farther away from the ulcer edge in 19/20 samples. Abundant expression of TIMP-3 was seen in all samples in fibroblasts, macrophages, and endothelial cells. When PG lesions of patients with underlying IBD were compared with those of nonIBD patients, TIMP-3 was decreased (1.6 vs. 2.4, $p<0.04$) in IBD patients. In acute wounds, TIMP-3 was generally not expressed by the migrating epidermal tip in keratinocytes, but farther down from the ulcer edge. TIMP-3 expression is very pronounced in fibroblast-like and endothelial cells of chronic wounds (Vaalamo et al. 1999) as well as in IBD stroma (Vaalamo et al. 1999), as we also found in PG. Unlike in mucosal and normally healing cutaneous wounds (Vaalamo et al. 1998, 1999), TIMP-3 protein was generally expressed by keratinocytes at the migratory front of PG. This suggests that it may inhibit in PG certain MMPs relevant for migration, and thus, retarding wound healing. Expression of MMPs in different categories of human wounds is summarized in Table 6.

Table 6. Expression of MMPs in different categories of human wounds. Results of this thesis are indicated in ***bold****, e = epithelium bordering the wound, s = stroma, (+) = a few cells, * = not migrating but proliferating epithelial cells, ´ = mRNA level, n.d. = not determined (Saarialho-Kere et al. 1996, 2002; Vaalamo et al. 1996, 1997, 1998, 1999; Saarialho-Kere 1998; Rechardt et al. 2000; Mirastschijski et al. 2002; Hieta et al. 2003; Impola et al. 2003, 2005; Kirkegaard et al. 2004; Ahokas et al. 2005).*

	Acute wound		**PG**		**IBD**	
	e	s	e	s	e	s
MMP-1	+	+	**+***	**++**	-´	++´
MMP-7	-	-	**-**	**(+)**	+	-
MMP-8	**-**	**(+)**	**-**	**+**	n.d.	n.d.
MMP-9	+	+	**(+)**	**++**	-	++
MMP-10	+	**(+)**	**++**	**++**	+´	+´
MMP-19	+*	+	n.d.	n.d.	**+***	**+**
MMP-26	+	-	**(+)**	**-**	**+**	**-**
MMP-28	+*	-	n.d.	n.d.	**+***	**-**
TIMP-1	-	+	**-**	**+**	-´	++´
TIMP-3	+*	+	**++**	**++**	-´	++´

5.10 MMP-21 is upregulated in pancreatic adenocarcinoma and expressed in low levels in PANC-1, BxPC-3, and AsPC-1 cell lines (V)

Expression of *MMP-21* protein was detected in 22/25 adenocarcinoma tissue samples and in 4/18 control samples. Staining was significantly more intense in tumor samples than in adjacent healthy tissue (1.2 vs. 0.22, $p<0.0000005$). Carcinoma cells were positive in 16/25 cases; 1/4 grade III, 13/18 grade II, and 2/3 grade I samples expressed MMP-21. MMP-21 concentrated in the central regions of the tumors and was not prominent at the invasive front. In cancer samples, the surrounding normal ductal structures were immunopositive in 6/25 samples. Only 2/25 samples were positive for MMP-21 in inflammatory cells. In fibroblasts, 3/25 samples showed positivity for MMP-21. As MMP-7 overexpression is considered a metastatic and prognostic marker in pancreatic cancer (Yamamoto et al. 2001; Li et al. 2005), we used MMP-7 as a positive control. It was detected in 9/9 studied adenocarcinoma samples in actual cancer cells, and macrophages were immunopositive in 5/9 adenocarcinoma samples. In immunopositive cancer cells, MMP-21 partly colocalized with MMP-7, while MMP-26 did not colocalize with MMP-21. MMP-21 expression diminished from T2 towards T4 (T2 1.5 vs. T4 0.5, $p<0.05$). The overall intensities of MMP-21, MMP-26, or TIMP-4 stainings did not correlate with survival time. In control samples, immunopositive structures were normal ducti and occasional macrophages.

In cultured cells, *MMP-21* was expressed basally at low levels in PANC-1 (34 cycles), BxPC-3 (34 cycles), and AsPC-1 (34 cycles). Its expression was induced 1.8-fold by EGF (10 ng/ml) in PANC-1 cells. In AsPC-1 cells, TNFα (10 ng/ml and 50 ng/ml) and TGFβ1 (5 ng/ml and 20 ng/ml) consistently downregulated the expression of MMP-21 0.6-fold and 0.7-fold, respectively. In the BxPC-3 cell line, cytokines did not influence MMP-21 levels. MMP-21 protein was also detected in the cytoplasm of PANC-1, BxPC-3, and AsPC-1 cells with immunostaining.

MMP-21 was found in cancer cells of well and moderately differentiated pancreatic adenocarcinomas, suggesting that it disappears from the most aggressive and dedifferentiated tumor cells. MMP-21 was not detected in neutrophils, like in esophageal SCCs (Ahokas et al. 2006), but was occasionally present in fibroblasts, in agreement with previous findings in certain skin disorders (Skoog et al. 2006). MMP-21 expression diminished from T2 towards T4 and was not associated with the presence of metastatic lymph nodes or with alterations in CA19-9 values. EGF, linked to poor survival (Garcea et al. 2005), was the only cytokine consistently able to induce MMP-21 in the cell line with a high metastatic ability, PANC-1. TGFβ1, associated with better prognosis and lower grade tumors (Garcea et al. 2005), did not upregulate MMP-21. The expression of TNFα mRNA in peripheral blood is upregulated in patients with pancreatic cancer and normalized after the pancreatic tumor is removed (Ariapart et al. 2002). In this study, TNFα downregulated the expression of MMP-21 only in the well-differentiated cancer cell line, AsPC-1, which suggests that more dedifferentiated cancer cells do not respond to this inflammatory cytokine by downregulating MMP-21 gene expression. MMP-21 mRNA was also expressed basally in the BxPC-3, a cell line with low metastatic potential.

MMP-21 is upregulated in esophageal and cutaneous SCCs (Ahokas et al. 2003, 2006). It is not induced in vitro in esophageal cancer cells by TGFβ1, PDGF, VEGF, bFGF, EGF, IFNγ, IL-1β, TNFα, retinoic acid, estrogen, fibronectin, laminin-1, or collagens I or IV

(Ahokas et al. 2006). In keratinocytes, MMP-21 is upregulated by TGFβ1, but not by PMA, EGF, IGF, bFGF, TNFα, or VEGF (Ahokas et al. 2003). In this study, MMP-21 was expressed basally at low levels and tissue data suggested it to disappear in the most poorly differentiated cells. Furthermore, MMP-21 was not expressed in apoptotic, LN-5-, β-catenin-, or Ki-67-positive cells in esophageal SCC (Ahokas et al. 2006). Therefore, MMP-21 might not be the key player in invasion, but rather induced at some point of dedifferentiation; however, confirmation of this requires a larger number of tumors, including the corresponding metastatic lymph nodes. Our results in melanoma suggest that expression of MMP-21 may serve as a marker of malignant transformation of melanocytes and is not associated with the presence of micrometastases (Kuivanen et al. 2005). MMP-21 is very differently regulated than older MMPs in macrophages and fibroblasts. It may participate in stromal remodeling and regulation of inflammation also in pancreatic cancer. Based on the structure and putative promoter sequence of MMP-21, it could be therapeutically manipulated by retinoids and furin inhibitors in pancreatic cancer (Skoog et al. 2006).

5.11 MMP-26 expression is associated with metastatic potential in pancreatic adenocarcinoma (V)

MMP-26 protein was detected in 13/25 adenocarcinoma samples and in 2/18 control samples, with staining being more intense in tumor samples (0.72 vs. 0.11, $p<0.001$). Positivity was observed in carcinoma cells in 10/25 samples; 2/4 grade III, 5/18 grade II, and 3/3 grade I samples expressed MMP-26. MMP-26 was generally found at the edges of the tumor islands, but not at the invasive front. MMP-26 was significantly more often expressed in tumors having metastatic lymph nodes (N0 0.27 vs. N1 1.07, $p<0.006$). The surrounding normal ducti and blood vessel walls were immunopositive in 12/25 cancer samples. Elastic fibers surrounding blood vessels were positive in 4/25 samples, agreeing with previous results in different skin disorders (Ahokas et al. 2005; Skoog et al. 2006). Only 4/25 samples showed positivity for MMP-26 in fibroblast/macrophage-like cells. In immunopositive cancer cells, MMP-26 colocalized with TIMP-4. In control samples, immunopositive structures were normal ducti. The malignant insulinomas were negative. In cultured cells, MMP-26 was not produced basally in any of the three pancreatic cancer cell lines, and none of the tested cytokines consistently upregulated MMP-26 mRNA in these cells.

MMP-26 can theoretically influence the behavior of pancreatic cancer by activating MMP-9 (Uria and López-Otin 2000). In several cancer types, MMP-26 has been shown to decrease progressively with loss of histological differentiation (Pilka et al. 2004; Lee et al. 2006). However, in esophageal SCC, MMP-26 overexpression correlates strongly with depth of invasion, lymph node metastases, and distant metastases (Yamamoto et al. 2004). In our material, MMP-26 was expressed by cancer cells in 52% of the adenocarcinomas examined. Positivity was found in moderately differentiated but not in poorly differentiated cells. In immunopositive cancer cells, MMP-26 colocalized with TIMP-4, as seen previously in breast cancer (Zhao et al. 2004). As in esophageal cancer cells (Ahokas et al. 2006), MMP-26 was not detected basally in three pancreatic cancer cell lines, and none of the cytokines tested was able to induce expression at detectable levels. Estrogen is a potential inducer of MMP-26 expression since the promoter region of MMP-26 contains an estrogen-responsive element (Pilka et al. 2004). Indeed MMP-26 has been implicated as an important factor regulating the proteolytic activity in estrogen-dependent

hyperplastic and malignant tissues (Li et al. 2006), but at least in esophageal SCC cells estrogen or dexamethasone were unable to upregulate its expression (Ahokas et al. 2006).

MMP-26 is also associated with migration of epithelial cells (Ahokas et al. 2005), and in our PG, study MMP-26 was increased in patients with immunosuppression. In pancreatic cancer, MMP-26 expression had a trend to increase from T1 to T4. MMP-26 was significantly more often expressed in primary tumors possessing metastatic lymph nodes. In fact, none of the node-negative samples had MMP-26-positive cancer cells. In our colon carcinoma study, MMP-26 was found bound to BM; hence, the speculation of MMP-26 having invasive potential is justified. Furthermore, MMP-26 gene expression is elevated in migrating breast tumor-associated endothelial cells in response to oxytocin induction (Cassoni et al. 2006). This suggests an even stronger role of MMP-26 in invasion and metastasis. Since MMP-26 has been associated with estrogen-dependent tumors (Li W et al. 2004; Satake et al. 2006) and several pancreatic cancer cell lines have estrogen receptors (Konduri and Schwarz 2007), studies with pancreatic cancer cells treated with estrogen or corticosteroids are warranted. Moreover, a higher number of tumors with corresponding metastatic lymph node specimens are required to confirm whether MMP-26 serves as a marker of metastasis in pancreatic cancer.

5.12 TIMP-4 is downregulated along with cellular dedifferentiation in pancreatic adenocarcinoma in vitro and in vivo (V)

TIMP-4 protein was detected in 22/25 adenocarcinoma samples and in 7/18 control samples, and staining was more intense in tumor samples (1.32 vs. 0.56, $p<0.003$). Carcinoma cells were positive in 15/25 samples; 3/4 grade III, 10/18 grade II, and 2/3 grade I samples expressed TIMP-4. When cellular atypia arose, TIMP-4 immunostaining decreased, but overall expression increased with TIMP-4 as cells became more undifferentiated (controls 0.56 vs. intermediately differentiated 1.28, $p<0.008$ and poorly differentiated 1.75, $p<0.007$). In cancer samples, the surrounding normal ducti and islets of Langerhans were immunopositive in 10/25 samples. TIMP-4 was not detected in inflammatory cells or endothelial cells, but immunopositive fibroblasts were seen in 4/25 samples. In immunopositive cancer cells, TIMP-4 partially colocalized with MMP-7 and MMP-26. In control samples, immunopositive structures were normal ducti and islets of Langerhans. In several controls (5/18) and carcinomas (4/25), the islets of Langerhans showed a positive immunosignal. TIMP-4 expression tended to diminish from T2 to T4, but this did not reach statistical significance. TIMP-4 expression correlated with increased CA19-9 values: (mean values 325-581-830, corresponding to immunograding 0-1-2). TIMP-4 was not expressed by malignant cells in insulinomas.

TIMP-4 was expressed basally in PANC-1 (35 cycles), BxPC-3 (29 cycles), and AsPC-1 (25 cycles) cells. Its expression was induced 2.7-fold by EGF (10 ng/ml) in BxPC-3 cells and 2.9-fold by TGFβ1 (20 ng/ml) in PANC-1 cells. In AsPC-1 cells, IFNγ (10 ng/ml and 25 ng/ml) consistently downregulated the expression of TIMP-4. TIMP-4 protein was detected in PANC-1 and AsPC-1 cells by immunohistochemistry.

TIMP-4 was detected in cancer cells in 60% of pancreatic adenocarcinoma samples, decreasing with cellular atypia. Immunopositive fibroblasts were occasionally seen, in agreement with results obtained in small-cell lung cancer (Michael et al. 1999). TIMP-4

expression tended to diminish from T2 to T4, and its expression correlated with increased CA19-9 values. Unlike observations of other TIMPs in pancreatic cancer (Gress et al. 1995; Jones et al. 2004), a tendency of TIMP-4 downregulation related to aggressiveness was noted, agreeing with a recent report (Fernandez-Figueiras et al. 2007). Furthermore, TIMP-4 mRNA was least expressed in the most undifferentiated pancreatic cancer cell line, PANC-1, while higher expression levels were detected in the less aggressive cell lines, AsPC-1 and BxPC-3. TIMP-4 is expressed in a subset of cells in islets of Langerhans, as observed with MMP-2, MMP-9, TIMP-1, and TIMP-2 (Tomita and Iwata 1997). Steroid hormones influence TIMP-4 expression in breast (Span et al. 2004) and endometrial cancers (Pilka et al. 2004). Its expression was lower in malignant endometrial tumors than in normal or hyperplastic endometrium (Pilka et al. 2004), in breast ductal carcinoma in situ than in infiltrating ductal carcinoma (Zhao et al. 2004), and in malignant chorioncarcinoma cells than in normal cytotrophoblasts. However, TIMP-4 is reported to be upregulated in cervical cancer compared with the corresponding normal tissue (Lizarraga et al. 2005) and also in papillary renal cell cancer (Hagemann et al. 2001), consistent with our observation in normal pancreatic tissue versus cancerous tissue. TIMP-4 is a potent inhibitor of MMP-26. TIMP-4 was expressed more in cancer samples than MMP-26 overall, but both of these decreased along with cellular dedifferentiation. TGFβ1 induced TIMP-4, which is in line with the finding that this cytokine generally upregulates TIMPs (Overall et al. 1989; Birkedal-Hansen 1993; Garcea et al. 2005). IFNγ is expressed in pancreatic cancer tissue, but not in pancreatic cancer cell lines (Andrianifahanana et al. 2006), suggesting that it is more likely secreted by inflammatory or fibroblast-like cells than by cancer cells. T-cells express less IFNγ in a tumoral microenvironment than normally (Liyanage et al. 2002). IFNγ induces apoptosis in pancreatic cancer cell lines AsPC-1, Capan-1, and Capan-2 (Detjen et al. 2001). IFNγ downregulated TIMP-4 expression in the well-differentiated cancer cell line, AsPC-1, but also in PANC-1 and BxPC-3 cell lines, but these results were not statistically significant. This may be an important factor in the transition phase of invasive cancer since MMP-26 is now suggested to be a candidate marker for invasive cancer, and reduction of MMP-26 inhibition could be the crucial trigger-point for invasion.

*Table 7. Summary of MMPs. Results of this thesis are indicated in **bold**. e = epithelium, s = stroma, n.d. = not determined, BM = basement membrane, ECM = extracellular matrix, - = no positive cases or very low number, (+) = moderate number of positive cases, + = over 50% of cases are positive (Mori et al. 1995; Yang et al. 2001; Ahokas et al. 2002 and 2003; Balaz et al. 2002; Salmela et al. 2002; Pender et al. 2006; **and this thesis**).*

	Normal intestine	**IBD**	**latent CD**	**NEC**	**PG**	**Colon cancer**	**Pancreatic cancer**
	e / s	e / s	e / s	e / s	e / s		
MMP-7	**+ / -**	**+/ -**	n.d.	**+ / -**	**- / (+)**	+	**+**
MMP-12	- / (+)	- / +	**- / +**	**- / +**	n.d.	+	+
MMP-19	**+ / -**	**+ / +**	**+ / +**	**(+)/ -**	n.d.	**-**	n.d.
MMP-21	+ / n.d.	n.d.	n.d.	n.d.	n.d.	+	**+**
MMP-26	**BM + / -**	**+ / -**	**- / +**	**+ / +**	**(+) / -**	**ECM +**	**+**
MMP-28	**+ / -**	**+/ -**	n.d.	n.d.	n.d.	**-**	n.d.

6. Conclusions

Matrix metalloproteinases have been implicated in tissue destruction, inflammation, and wound healing in different organs as well as in cancer initiation, growth, and metastasis. MMPs are in general the main ECM-degrading enzymes and their uncontrolled function can be devastating. During the past ten years several new members of the MMP family have been cloned. Their function and cellular localization have not yet been fully elucidated. Identification of distinct cell types that express certain MMPs is important for understanding their function – not only in certain diseases but also in experimental models. Knowing the cell type is the first step in understanding which MMP should be therapeutically inhibited.

This PhD study aimed to characterize the contribution of MMPs-12, -19, -21, -26, and -28, in particular, in intestinal inflammation, regeneration, and transformation. In IBD, MMP-19, which could be upregulated by the proinflammatory cytokine TNFα, was expressed in the proliferating epithelium and in some stromal macrophages and fibroblasts, suggesting its involvement in restoring the normal composition of intestinal epithelium and mucosa. We are the first to show that MMP-26 is found intracellularly in migrating enterocytes. It was also detected bound to the BM, suggesting that MMP-26 cleaves some BM-associated proteins. MMP-28 was seen in proliferating epithelium, but not in migrating or inflammatory cells. MMP-28 could therefore be involved in normal tissue turnover, like MMP-19.

While our NEC study was in progress, MMP-3 was reported to be responsible for the extensive tissue injury in infants with NEC (Pender et al. 2003). MMPs are a large and multifunctional family of tissue-degrading proteases. There is no point in elevating one MMP over the other before thorough investigations involving several potential MMPs are carried out. We expanded the information on the MMP profile at the tissue level, and our results suggest that MMPs-1, -7, and -9 in the epithelium and stroma, and MMPs-12 and -26 in macrophages, in addition to MMP-3, may be major factors in tissue destruction and remodeling in NEC. Importantly, this is the first work describing MMP-26 protein in macrophages.

Several MMPs have previously been linked to tissue alternations occurring in the celiac intestine and dermatitis herpetiformis. In this study, we found MMP-12 to be the most promising candidate as a marker for latent celiac disease, for it was positive in initial biopsies in later diagnosed CD patients and in patient samples with elevated levels of CD-associated serum markers. The number of later diagnosed CD patients was not high, 2/28, and thus, a larger patient population is needed to confirm this finding. Nevertheless, children with potential CD should be carefully followed up if they show MMP-12 positivity in a tissue sample, yet fail to fulfill the diagnostic criteria for CD. Furthermore, this is the first study examining MMPs in the gut of T1D patients to demonstrate increased expression of MMPs-1, -3, and -12 in the intestinal stroma of children with diabetes even when no significant histological changes are observed.

In PG, an extraintestinal manifestation of IBD, several MMPs are involved in tissue destruction. Abundant expression of MMPs-9 and -10 and TNFα was seen in the stroma, and their expression may contribute to the degradation of the provisional matrices needed for fibroblast and keratinocyte migration. MMPs-1 and -26 were in most cases absent from the migrating tip, and this may retard epithelial repair. We are the first to demonstrate

abundant expression of TNFα in PG at the tissue level (in 96% of studied patients). Inhibiting TNFα could reduce MMP-associated inflammation and cure the chronic wound. In fact, the TNFα inhibitor, infliximab, has already given promising results. The restrictive factor in PG studies is its relatively small incidence rate. Although several IBD patients suffer from PG, the prevalence is fairly low in Finland.

MMPs are connected to cancer invasion, and thus, we studied the role of several novel MMPs in colorectal and pancreatic adenocarcinomas. MMP-26 was diminished in both cancer types in dedifferentiated cancer cells, but it is suggested to be involved in metastatic potential since it is expressed in the nearby ECM surrounding cancer islets in colon carcinoma and is strongly expressed in patients with nodal metastases in pancreatic cancer. These results require, however, confirmation in a larger patient population. TIMP-4, a potent inhibitor of MMP-26, is also downregulated along with cellular dedifferentiation in pancreatic cancer, and this was also confirmed in cultured pancreatic cancer cells. MMP-21 was associated with differentiation in pancreatic cancer, and its expression did not correlate with tumor stage, metastases, or survival time. MMPs-19 and -28 are not involved in tumor progression in colorectal carcinoma.

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