Matrix Metalloproteinases in Gastric Inflammation and Cancer

Clinical Relevance and Prognostic Impact

Frank Kubben

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Abbreviations

ABC, avidin biotin complex
ADAMs, a disintegrin and metalloproteinases
ADAMTSs, a disintegrin and metalloproteinases with thrombospondin motifs
APMA, *p*-aminophenylmercuric acetate
ARMS, amplification refractory mutation system
BIA, bioactivity assay
BSA, bovine serum albumin
CI, confidence interval
ECM, extracellular matrix
ELISA, enzyme-linked immunosorbent assay
EMMPRIN, extracellular matrix metalloproteinase inducer
ERK, extracellular signal-regulated kinase
F, female
FDA, Food and Drug Administration
HNL, human neutrophil lipocalin, also named NGAL
Hp, *Helicobacter pylori*
HR, hazard ratio
HRP, horseradish peroxidase
kDa, kilo Dalton
M, male
MALT, gastric mucosa associated lymphoid tissue
MMPs, matrix metalloproteinases
MPO, myeloperoxidase
mRNA, messenger ribonucleic acid
MT-MMPs, membrane-type matrix metalloproteinases
NA, not applicable
ND, not defined
NGAL, neutrophil gelatinase-associated lipocalin, also named HNL
NS, non significant
NSAIDs, non-steroidal anti-inflammatory drugs
PBS, phosphate-buffered saline
PCR, polymerase chain reaction
PUMP-1, putative matrix metalloproteinase-1 (MMP-7)
RFLP, restriction fragment length polymorphism
S-2444, pyro-Glu-Gly-Arg-*p*-nitroanilide
SEM, standard error of the mean
SNP, single nucleotide polymorphism

TBS, tris-buffered saline
TIMPs, tissue inhibitors of metalloproteinases
TNM, tumour node metastasis
VEGF, vascular endothelial growth factor
WHO, World Health Organisation

CHAPTER 1

General introduction

Introduction

Helicobacter pylori infection of the stomach causes a chronic gastritis that is associated with the development of peptic ulcer disease and gastric cancer. Chronic inflammation and malignancy are diseases that are accompanied by excessive degradation of the extracellular matrix. Matrix metalloproteinases are zinc-dependent proteinases that are involved in these processes because of their capability of digesting various structural components of the extracellular matrix. The list of their known substrates has expanded over the years to a broad range of extracellular proteins including other proteinases, proteinase inhibitors, receptors, clotting factors, cytokines, growth factors and chemotactic molecules. Enhanced matrix metalloproteinase levels and activities have been described in *Helicobacter pylori*-induced gastritis and in gastric cancer. In this thesis several studies are described that assessed the putative role of matrix metalloproteinases in chronic *Helicobacter pylori*-induced gastritis and gastric cancer.

Extracellular proteolysis

Extracellular proteolysis plays an important role in cell-cell and cell-matrix interactions of physiological processes like mammary gland involution, ovulation, blastocyst implantation, cellular migration and angiogenesis, but also in pathological conditions like inflammation as well as invasion and metastasis of malignant tumours [1-8]. Whereas in physiological conditions this proteolysis is controlled and self-limiting, in inflammation and metastasis there appears to be an excessive or unbalanced production of proteolytic enzymes. These proteolytic enzymes play an important role in the remodelling and breakdown of the extracellular matrix (ECM). The ECM, consisting of basement membranes and interstitial stroma, is composed of a large number of components that interact with each other and with the different cell types present. Collagens are the most abundant ECM constituents besides adhesive glycoproteins like laminin, fibronectin, elastin and proteoglycans-glycosaminoglycans like hyaluronic acid and heparan sulphate [9-11]. Proteinases can be classified in four main groups: 1) Cysteine proteinases (e.g., cathepsin-B, -H, -L and -N); 2) Aspartyl proteinases (e.g., cathepsin-D); 3) Serine proteinases (e.g., cathepsin-G and -E, elastase, kallikrein, thrombin, trypsin, plasmin, plasminogen activators); and 4) Metalloendopeptidases. These metalloendopeptidases include the thermolysins, insulinases and metzincins (zinc-dependent proteinases). Metzincins can subsequently be subdivided into matrix metalloproteinases (MMPs, matrixins), adamalysins (ADAMs; <u>a</u> <u>d</u>isintegrin <u>a</u>nd <u>m</u>etalloproteinase<u>s</u>, and ADAMTSs; <u>a</u> <u>d</u>isintegrin <u>a</u>nd <u>m</u>etalloproteinases with <u>t</u>hrombo<u>s</u>pondin motifs), astacins and serralysins [12-14].

Matrix metalloproteinases

The matrix metalloproteinase (MMP) gene family consists of a group of proteolytic enzymes capable of degrading components of the ECM during physiological processes like pregnancy, parturition [15], development, growth and wound-healing [16], as well as in pathological conditions like rheumatoid arthritis [17], pulmonary emphysema [18], osteoarthritis [19], skin disorders [20] and malignancy [21]. MMPs share the following functional properties: 1) they contain a zinc ion at their active site and can be inhibited by chelating agents; 2) they are almost all secreted in a latent zymogen form that needs activation by partial proteolytic cleavage to become active; 3) they are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs); and 4) they share common amino acid sequences and cleave at least one component of the ECM [22]. Depending on their structure and substrate preference, the MMP family is divided into collagenases (MMP-1, -8, -13 and -18), stromelysins (MMP-3, -10, -11 and -28), matrilysins (MMP-7 and -26), gelatinases (MMP-2 and -9), elastases (MMP-12, -19 and -20) and membrane-type MT-MMPs (MMP-14, -15, -16, -17, -24 and -25). Some relevant characteristics of the specific MMPs and TIMPs studied as described in this thesis are summarized in Table 1 and discussed below.

Collagenases. The collagenases can degrade structural type I to III collagens only. Neutrophil collagenase or collagenase-2 (MMP-8) is one of the collagenases regarded as being synthesized exclusively by polymorphonuclear neutrophils before emigration from the bone marrow. In polymorphonuclear leucocytes it is stored in and released from secretory granules and its expression is stimulated by tumour necrosis factor-α. In addition, MMP-8 mRNA has been detected in mononuclear fibroblast-like cells in rheumatoid synovial fibroblasts and endothelial cells. Doxycycline has been shown to down-regulate MMP-8 induction, at both the mRNA and protein levels [17].

Matrilysins. Matrilysin (MMP-7) or putative matrix metalloproteinase-1 (PUMP-1) lacks a specific extracellular matrix-binding domain and is therefore the smallest of the MMP gene family, with a molecular weight in its inactive form of 28 kDa. The zymogen is activated by 4-aminophenylmercuric acetate, trypsin, plasmin and stromelysin-1 (MMP-3), but not by tissue collagenase (MMP-1), gelatinase-A (MMP-2) nor gelatinase-B (MMP-9). MMP-7 can activate pro-MMP-1 and pro-MMP-9 but not pro-MMP-2 nor pro-MMP-3. It has strong stromelysin-like activity and degrades insoluble elastin, type IV collagen, laminin-1, fibronectin, proteoglycan and gelatins [27].

Gelatinases. Gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are originally called gelatinases, enzymes which degrade denatured collagens (gelatin), although both gelatinases can degrade native collagens including type IV (basement membrane) and type V collagen and elastin as well. MMP-2 has been demonstrated in a variety of normal and malignant cells whereas MMP-9 is mainly expressed by alveolar mac-

Table 1 - Characteristics of the proteinases studied

Proteinase	MMPs				TIMPs		Lipocalins
Subgroup	Collagenase	Gelatinases		Matrilysins			
Nomenclature	MMP-8	MMP-2	MMP-9	MMP-7	TIMP-1	TIMP-2	Lipocalin-2
Common name	Neutrophil collagenase	Gelatinase-A	Gelatinase-B	Putative matrix metalloproteinase (PUMP-1); Matrilysin			Neutrophil gelatinase-associated lipocalin (NGAL)
Substrate specificity	Collagen I, II, III, VII, X	Gelatin, collagen type IV and V, elastin, laminin	Gelatin, collagen type IV and V, elastin, laminin	Gelatin, fibronectin, laminin, collagen type IV, procollagenase, TNF-α precursor	Pro-MMP-9	Pro-MMP-2	Bacterial formyl-peptide FMLP; MMP-9
Molecular mass (kDa)	75	72	92	28	28.5	21	25
Molecular mass of active species (kDa)	55	62; 64	67; 82	19			
Physiological activators	Serine proteases	MT-MMP; type 1 collagen	Serine proteases; MMP-2; MMP-7		EGF; IL-6; IL-1; IL-1β; Epo; TGF-β		
Native inhibitor	TIMP-1; TIMP-2	TIMP-2 > TIMP-1	TIMP-1 > TIMP-2	TIMP-1; TIMP-2			
Expression	Inducible	Constitutive	Inducible	Inducible	Inducible	Constitutive	Inducible
Localization	Neutrophils	Fibroblasts	Neutrophils	Epithelial cells; tumour cells	Fibroblasts; Epithelial and endothelial cells; tumour cells		Neutrophils
Reference	23	24	24	23	25		26

rophages, monocytes, keratinocytes, polymorphonuclear leucocytes and malignant cells [28, 29].

Tissue inhibitors of metalloproteinases

Tissue inhibitors of metalloproteinases (TIMPs) are ubiquitous and naturally occurring inhibitors of MMPs that inhibit the MMP proteolytic activity by forming noncovalent 1:1 stoichiometric complexes resistant to heat denaturation and proteolytic degradation [for review see 25]. Up to now, four TIMPs have been described, TIMP-1, -2, -3 and -4, with molecular weights ranging from 21 to 28.5 kDa. TIMPs differ in solubility, interaction with proenzymes (pro-MMPs) and regulation of expression. Whereas TIMP-1, -2 and -4 are present in soluble forms, TIMP-3 is tightly bound to the matrix. Apart from binding to MMPs, TIMPs are also able to form complexes with the pro-MMPs influencing the MMP activation process. TIMP-2 is a constitutive protein, whereas TIMP-1 expression is influenced by external stimuli including growth factors, serum, phorbol esters, cytokines and erythropoietin. It has become apparent that apart from regulation of MMP activity, TIMPs are also involved in various other biological processes including cell-growth, apoptosis and angiogenesis. For example, TIMP-1 and -2 have been shown to potentiate the effect of erythropoietin on erythroid stem cell proliferation and differentiation. Moreover, TIMP-1 and -2 are able to induce the growth of normal and malignant cells and are associated with resistance against apoptosis in malignant cells. In addition, TIMP-1 has been shown to potentiate steroidogenesis. Moreover, TIMPs are also able to inhibit angiogenesis, partly by MMP inhibition, and are involved in embryogenesis by controlling the MMP-mediated remodelling of the extracellular matrix during blastocyst implantation. In tumours of various origins, downregulation of both TIMP-1 and -2 has been associated with increased invasiveness, while overexpression was associated with reduced tumour growth and metastasis.

Detection of matrix metalloproteinases

MMPs can be detected by various techniques including zymography, *in situ* zymography, enzyme-linked immunosorbent assay (ELISA), immunohistochemistry, mRNA *in situ* hybridization and quantitative RT-PCR analysis, Western blotting and quantitative activity assays, including radiolabelled collagen substrate degradation assays and bio immuno assays (BIAs). Gelatin zymography allows quantification of the active and the pro-enzyme form but is primarily suitable for measurement of gelatinases [30] and does not provide information at the cellular level. This disadvantage has been

overcome with the introduction of *in situ* zymography, that enables visualisation of activity of the gelatinases MMP-2 and -9 [31-34] and of MMP-7 [35] on frozen tissue sections. MMPs and their TIMPs can be localized by immunohistochemistry and mRNA *in situ* hybridization analysis without possibilities for quantification and detection of enzyme activity [36, 37]. In general, most ELISAs measure the grand total of pro-enzyme, active- and inhibitor-complexed forms of the respective MMP [38]. Enzymatic activities of MMPs can also be measured in blood samples and tissue homogenates using specific biochemical immunosorbent activity assays (BIA) [39, 40].

Regulation of matrix metalloproteinases

MMPs are tightly regulated at different levels, including gene expression, secretion, activity, and clearance [41].

1) Gene expression. The expression of most MMPs is regulated at the transcriptional level, by growth factors (e.g., epidermal growth factor and transforming growth factor-β), cytokines (e.g., tumour necrosis factor-α and interleukin-1), hormones [42-44], bacterial endotoxins, stress and oncogene activation [45, 46]. Apart from soluble factors, also cell-cell and cell-matrix interactions influence the expression of MMPs. For example, extracellular matrix metalloproteinase inducer (EMMPRIN), formerly called tumour cell-derived collagenase stimulatory factor, is a glycoprotein located on the outer surface of human tumour cells which interacts with fibroblasts to stimulate expression of several matrix metalloproteinases in fibroblasts [47, 48]. Various single nucleotide polymorphisms (SNPs) in gene promoter regions of MMPs have been shown to influence transcriptional activity resulting in altered protein levels. The -1306 C/T transition in the MMP-2 promoter sequence, for example, disrupts a Sp-1 binding site resulting in remarkably decreased promoter activity [49]. The -1562 C/T transition in the promoter region of the MMP-9 gene, on the other hand, results in higher promoter activity. This is due to preferential binding of a putative transcription repressor protein to the C allelic promoter [50].

2) Secretion in the latent form. MMPs are secreted in a latent proenzyme form, with the exception of MMP-11, MMP-28, and the membrane bound MT-MMPs. The other MMPs need to be activated in order to interact with the extracellular matrix. Several proteolytic activators, including the plasminogen activator/plasmin system, kallikreins, neutrophil elastase and trypsin, are involved in the activation of MMPs [51]. Unlike other MMPs, pro-MMP-2 is constitutively expressed by many cell types and activation occurs at the cell surface [52]. This activation appears to involve an interaction with MT1-MMP and TIMP-2 to form a membrane-bound complex that regulates the activation of pro-MMP-2 [53]. Pro-MMP-9 is not constitutively expressed

and its production and secretion can be induced and modulated by various factors. For example, pro-MMP-9 is synthesized by differentiating neutrophils in the bone marrow, stored in specific granules of circulating neutrophils, and released following neutrophil activation by inflammatory cytokines [54, 55].

3) Regulation of activity. TIMPs, the primary inhibitors of MMPs, form inhibitory complexes with most active MMPs [56, 57]. TIMP-1 also binds to pro-MMP-9, whereas TIMP-2 forms complexes with pro-MMP-2 [58]. TIMPs inhibit MMPs locally whereas alpha-2-macroglobulin, an abundant plasma protein, acts as a general non-specific endoproteinase inhibitor [59]. Recently, the transformation suppressor factor RECK, a membrane-anchored glycoprotein, was found to contain 3 protease inhibitor-like domains that negatively regulate MMP-2, -9 and MT1-MMP activity and inhibits tumour invasion and metastasis. Furthermore, down-regulation of RECK by oncogenic signalling leads to the excessive activation of MMPs thereby promoting malignant behaviour of cancer cells. In several types of tumours, a positive correlation between RECK expression and survival of the patients has been described [60-62].

4) MMP catabolism and clearance. Although MMPs are found in urine, the degradation and excretion pathways of MMPs and TIMPs in the body have not been fully elucidated [63]. Little is known about autoproteolysis of active MMPs, but certain cleavages clearly diminish MMP-activity [54]. Some MMPs are also found in association with accessory proteins, like lipocalins, which could serve as protection against autolysis.

Human neutrophil lipocalin

Lipocalins are a group of small extracellular proteins with great diversity at the sequence level. The lipocalins are member of an overall structural superfamily: the calycins. The other groups are the fatty-acid-binding proteins, a group of metalloprotease inhibitors, triabin and the avidins [64]. Lipocalins bind to a spectrum of small hydrophobic molecules and to specific cell-surface receptors and form complexes with soluble macromolecules. Lipocalins are supposed to function as transport proteins. They appear to be involved in biological processes like retinol transport, invertebrate cryptic coloration, olfaction, pheromone transport and prostaglandin synthesis. They also play a role in the regulation of cell homeostasis, the modulation of the immune response, and, as carrier proteins, act in the clearance of endogenous and exogenous substances [65]. Neutrophil gelatinase-associated lipocalin (NGAL), also named human neutrophil lipocalin (HNL), siderocalin or lipocalin-2 (lcn2), is a 25 kDa glycosylated protein constitutively expressed in myelocytes and stored in secondary granules of human neutrophils [66]. NGAL is present as a monomer, homodimer, or

as a heterodimer with neutrophil gelatinase [67]. It is highly induced in epithelial cells during inflammation or malignant conditions [68-74]. Because accumulation and infiltration by neutrophils is a prominent feature of the inflammatory process in ulcerative colitis, it has been suggested that NGAL may serve as a specific marker of intestinal neutrophil activation in ulcerative colitis [75]. In neutrophils, NGAL colocalizes with lactoferrin, whereas the gelatinases are localized in specific gelatinase granules [67, 76]. Interaction of NGAL with activation and enzymatic activity of gelatinase could initially not be shown [77]. The expression of NGAL in epithelial cells is totally dependent on NF-κB and also depends on a NF-κB-binding co-factor that is induced by interleukin-1β but not by tumour necrosis factor-α and is required for transcription of the NGAL gene [78]. It has been shown that NGAL can act as a bacteriostatic agent by sequestering siderophore bound iron and preventing its uptake by microorganisms [73, 79, 80]. Therefore, NGAL seems to play a role also in the innate immunity by reducing the availability of iron for microbial growth [78].

Helicobacter pylori infection and gastric neoplasia

Gastritis is inflammation of the stomach and is caused by infectious agents, drugs, and autoimmune and hypersensitivity reactions. Gastritis in childhood is very common in non-industrialized countries but uncommon in the Western world. The prevalence of gastritis increases with age, reaching a prevalence of approximately 60% in industrialized to 100% in non-industrialized countries at the age of 60 [81-83]. The most frequent cause of (chronic) gastritis is *Helicobacter pylori* (*H. pylori*), a Gram-negative bacterial species that preferentially colonizes human gastric mucosa resulting in a chronic gastritis [84, 85]. After eradication of *H. pylori,* the gastric mucosa is restored to normal [85]. In the majority of patients with peptic ulcer disease *H. pylori* is the causative factor [86-88] and eradication of *H. pylori* prevents recurrence of peptic ulcers [89]. *H. pylori* has also been associated with gastric carcinoma [90], gastric mucosa associated lymphoid tissue (MALT), lymphoma [91] and Ménétrier's disease [92]. *H. pylori* gastritis progresses gradually over the years from the non-atrophic form into the atrophic form. Atrophic gastritis and intestinal metaplasia are premalignant conditions for gastric cancer except for cancers of the gastrointestinal junction. Of gastric carcinomas, 80% are related to *H. pylori* gastritis. *H. pylori* infection is currently associated with an approximately two-fold increased risk of developing gastric cancer [93]. In addition to *H. pylori* gastritis, another 10% of gastric carcinomas are related to atrophic gastritis of the autoimmune type [94, 95]. A possible mechanism through which *H. pylori* infection could induce carcinogenesis is via the accompanying inflammatory response, which gives rise to the production of mutagenic substances like

nitric oxide [96]. Although *H. pylori* appears to be the most frequent cause of (chronic) gastritis [84, 85], only a minority of people who harbour this organism ever develop cancer. This process of gastric carcinogenesis seems to be influenced by differences in inflammatory response due to genetic diversity of both *H. pylori* isolates as well as host genes [97]. Four different genetic loci have been identified in the *H. pylori* genome with the potential to interact with host molecules that induce epithelial responses with carcinogenic potential: *CagA, VacA, BabA, and SabA*. *CagA*, derived from the cytotoxin associated gene, is injected in the host cells by the bacterium. *CagA*$^+$ strains enhance the risk for severe gastritis, atrophic gastritis, and distal gastric cancer compared with that incurred by *cag*$^-$ strains [98, 99]. One of the mechanisms involved is an enhanced NF-κB mediated interleukin-8 induction and inflammatory neutrophilic response in human gastric tissue by *H. pylori cagA*$^+$ strains compared to *cag*$^-$ strains [100]. *VacA* encodes a secreted bacterial cytotoxin that induces multiple structural and functional alterations in cells leading to gastric inflammation, haemorrhage, and ulcers [101, 102]. *H. pylori* strains with a type s1/m1 *vacA* allele are associated with enhanced gastric epithelial cell injury [103] and gastric cancer risk compared with *vacA* s2/m2 alleles [104]. *BabA2* encodes for an adhesin that binds the Lewis^b histo-blood-group antigen on gastric epithelial cells. The presence of *babA2* is associated with *cagA* and *vacA* s1 and *H. pylori* strains with all three genes induce the highest risk for gastric cancer [105]. Sialyl-Lewis^x antigen is a tumour antigen that is upregulated in gastric inflammation binding to gastric epithelial cells by the *H. pylori* adhesin *sabA* [106].

MMPs in gastrointestinal immunity, inflammation, infection and carcinogenesis

MMPs are tightly regulated and under normal conditions are involved in physiological tissue turnover and the host immune response. They play a role in matrix remodelling, the recruitment of inflammatory cells into the intestinal wall and other organs, cytokine and chemokine processing and defensin activation. MMP-9, for example, is secreted during neutrophil migration across the basement membrane, whereas TIMP-1 is able to inhibit this process [107]. MMP-3 is involved in the cellular immune response against intraluminal colonic pathogenic bacteria by facilitating the migration of T-helper lymphocytes into the intestinal lamina propria [108]. The gelatinases are also necessary for migration of dendritic cells out of the skin and of T-cells across the basement membrane [109]. Several MMPs, including the gelatinases, can release active tumour necrosis factor-α from the membrane-anchored precursor [110] and can both activate pro-interleukin-1β or inactivate active interleukin-1β [111, 112]. Defensins are MMP-7 activated antibiotic peptides that kill bacteria by membrane dis-

ruption. It has been shown that certain bacterial components can stimulate epithelial MMP-7 secretion, indicating its role in the early defence mechanism against infection [113].

Under pathological conditions, including gastrointestinal inflammation, infection and malignancy, enhanced levels and activities of MMPs have been described resulting in an imbalance in breakdown and remodelling of the extracellular matrix [114, 115]. In inflammatory bowel diseases, for example, enhanced expression of MMPs, either on the protein or mRNA level, or immunohistochemically, has been described to be associated with the severity of inflammation [116-118]. Protein and mRNA levels of MMP-2 and especially MMP-9 were markedly enhanced in inflammatory bowel tissues, with the highest concentrations in severely inflamed tissues. Polymorphonuclear leucocytes appeared to be the main source of MMP-9, whereas MMP-2 was predominantly located in the extracellular matrix [116]. Epithelial cells at the edge of gastrointestinal ulcers are strongly positive for matrilysin (MMP-7), probably because of their putative role in re-epithelization [119]. It has been demonstrated that activated lamina propria T-cells, for example elicited by luminal antigens, can cause a pathological chronic inflammatory response leading to intestinal damage by stimulating MMP secretion [120]. Enhanced MMP levels and activities have also been described in necrotising enterocolitis [121], celiac disease [122], collagenic colitis [123] and diverticulitis [124].

In infectious diseases, enhanced host MMP activity or decreased TIMP expression has been described in response to pathogens. In addition to inducing MMP secretion by host cells, it has been shown that bacterial pathogens are able to activate host pro-MMPs by secreting proteolytic enzymes themselves [125]. *H. pylori*, for example, produces several metalloproteases including one with MMP-3 like activity [126, 127].

In a number of human cancers enhanced expression of many MMPs, including MMP-1, -2, -3, -7, -9, -13 and -14, at the protein and mRNA levels or immunohistochemically, in both primary tumours and/or metastases has been associated with tumour progression and poor prognosis [128]. For instance, enhanced immunohistochemical expression of MMP-1, -7 and -13 has been associated with poor prognosis in colorectal cancer patients [129-131]. Enhanced expression of MMPs is found at the invasive front of tumours where malignant cells and stromal cells interact with each other and mutual induction of MMPs takes place. Several specific cellular mechanisms have been described that facilitate activation of local pro-MMPs and containment of MMP activity to the invasive front. These mechanisms include: 1) the expression of membrane-bound metalloproteinases; 2) the binding of soluble MMPs to membrane-bound docking factors; and 3) cell surface receptor-mediated activation of pro-MMPs. Pro-MMP-2, for example, can be activated at the cell membrane after forming a trimeric complex with TIMP-2 and MT1-MMP (MMP-14) [53].

Chronic inflammation is associated with enhanced cancer risk and both chronic inflammation and cancer are accompanied by enhanced MMP levels and activities [97]. Malignant cells, on their turn, secrete cytokines and MMPs, which stimulates influx of inflammatory cells to the tumour site and induces (neo)angiogenesis [132]. It has been shown, for instance, that MMP-9 is involved in tumour-induced angiogenesis by releasing Vascular Endothelial Growth Factor (VEGF) [133] with tumour-related inflammatory cells being the main source of MMP-9 [134].

MMPs in *H. pylori*-induced gastritis

Immunohistochemical studies on human gastric tissue have shown that MMP-9 immunoreactivity is predominantly expressed by inflammatory cells, including macrophages and fibroblasts [135, 136], by parietal cells [137], and to a lesser extent by epithelial cells with higher expression in *H. pylori* positive tissue compared to *H. pylori* negative tissue [135, 136]. Antral mucosa of *H. pylori* positive individuals showed approximately 20-fold higher MMP-9 activity compared to that of uninfected individuals, when measured by quantitative gelatin-zymography, probably by an increased number of macrophages containing a higher amount of MMP-9. Macrophages secrete MMP-9 in response to *H. pylori* [136, 138] and it has been demonstrated that *H. pylori* can induce activation of NF-κB in gastric epithelial cell lines leading to MMP-9 gene transcription [135].

In histologically normal, *H. pylori* negative human gastric tissue MMP-2 immunoreactivity was observed in parietal cells and to a lesser extent in epithelial cells [137]. MMP-2 activity was elevated in *H. pylori* positive individuals compared to uninfected individuals, but at lower levels than MMP-9 [136].

H. pylori positive individuals expressed higher levels of MMP-7 at the protein and mRNA levels in their antrum and corpus when compared to uninfected individuals. MMP-7 immunoreactivity in epithelial cells of *H. pylori* positive individuals was more intense than in uninfected persons [138, 139]. MMP-7 expression was strongly related to the infestation of cag^{+} *H. pylori* strains since MMP-7 expression was demonstrated in gastric epithelial cells in 80% of *cag*$^{+}$ colonized persons but in none of *cag*$^{-}$ or uninfected individuals. *Cag*$^{+}$ *H. pylori* strains augment the risk for gastric cancer. In *in vitro* studies the increased levels of MMP-7 in inflamed gastric mucosa appeared to be induced by *cag*$^{+}$ *H. pylori* strains dependent on activation of extracellular signal-regulated kinase (ERK) 1/2 mitogen–activated protein kinase [140]. *H. pylori* infection has also been reported to stimulate MMP-1, MMP-3, TIMP-3, and MMP-3/TIMP-3 complex formation in gastric epithelial cells and in gastric mucosa [141, 142]. A strong interaction of interleukin-1β and *H. pylori* on MMP-3 secretion has been found [142].

In *H. pylori*-induced ulcers higher concentrations of MMP-1 were found compared to NSAID (non-steroidal anti-inflammatory drug)-induced ulcers, possibly due to the anti-inflammatory effect of the NSAIDs used [143].

Gastric carcinoma

Despite the sharp decrease in the incidence rate of gastric cancer over the last 50 years [144, 145] gastric cancer is still the second most common cancer worldwide accounting for approximately 10% of all cancers and being responsible for approximately 12% of all cancer deaths [146-148]. In contrast to the decreased incidence rate of gastric cancer, the incidence rates of cancer of the distal oesophagus and of the cardia have increased significantly over the last decades [149]. In 1985, the age-standardized incidence rate per 100,000 population varied from 8.8 and 4.2 in North America to 74.8 and 35.2 in Japan, in men and women, respectively. The incidence rate for Western Europe in 1985 was 18.0 and 9.4 in men and women, respectively [146, 150]. The incidence rate in the Netherlands from 1989 until 1992 was 15.4 and 6.1 in men and women, respectively (World Standardized Rate) and in this period, 10,116 new cases of gastric cancer were registered indicating approximately 2,500 new cases annually [151]. In 1998, the incidence rate in the Netherlands ranged from 18 in men (1,392 new cases) to 10 in women (768 new cases) according to the Dutch Cancer Registry (www.kankerregistratie.nl).

The 5-year cumulative survival rate ranges from 91% in stage 1 (intramucosal) to 5% in stage 4 (metastatic) gastric cancer [152]. The overall 5-year survival rate ranges from 7.4% to 16.5% [153, 154]. In the southeast of the Netherlands, the relative 5-year survival rate of patients who underwent resection was 85% for stage I and 60% for stage II tumours [155]. Surgical resection remains the primary curative treatment option in gastric cancer with 5-year survival rates of 58%-78% and 34% reported for stage I and II disease, respectively [156]. Postoperative chemoradiotherapy, perioperative chemotherapy, and postoperative chemotherapy have been shown to decrease the risk for recurrence and to improve the outcome for patients fit to undergo these treatments [157]. The aetiology of gastric cancer seems to be multifactorial with different relative influence of causal factors in different geographical regions. Compared to U.S.-born individuals, immigrants had at least a 50% higher mortality from stomach cancer [158]. Epidemiological data suggest an increased risk for gastric carcinoma development in patients with *H. pylori*-associated gastritis [90, 159]. *H. pylori* infection was especially associated with non-cardiac carcinomas, and a stronger association was observed with diffuse rather than with intestinal-type tumours [160].

MMPs in gastric carcinoma

Immunohistochemical and *in situ* hybridisation studies, as well as quantitative methods like gelatin-zymography and activity assays, have demonstrated that gastric carcinomas contain enhanced amounts of MMP-1, -2, -3, -9 and TIMP-1 [161, 162], MMP-7 [163] and MT1-MMP [164]. Particularly MMP-1, MMP-7, MMP-9 and TIMP-2 were immunolocalized in carcinoma cells, whereas MMP-2 immunostaining was observed on advanced gastric carcinoma cells and correlated with vascular invasion by tumour cells [165, 166]. Pro-MMP-2 activation was present only on gastric carcinoma cells that expressed MT1-MMP, indicating MT1-MMP-assisted activation of pro-MMP-2 in human gastric carcinomas. The MMP-2 genotype appeared to influence the susceptibility to develop gastric cardiac adenocarcinoma [166].

MMP-7 was reported to be produced by gastric carcinoma cells and significantly associated with aggressive pathological phenotypes of gastric cancer [167]. In gastric carcinomas, it has been shown that expression of E1AF/PEA3 (ETV4), an ets-family transcriptional factor, able to transactivate multiple MMP genes, correlates well with MMP-7 expression [168].

H. pylori infection of gastric carcinoma cells was reported to increase mRNA expression and protein levels of MMP-9 [169]. MT1-MMP [170] and MMP-1 [171] immunoreactivity in human gastric carcinomas were also found to be associated with worse prognosis, whereas increased TIMP-2 expression seems to be correlated with prolonged survival [172].

Outline of the studies described in this thesis

In this thesis, several studies are described on the putative role of the matrix metalloproteinases MMP-2, -7, -8 and -9, the tissue inhibitors of metalloproteinases TIMP-1 and -2, and of the lipocalin neutrophil gelatinase-associated lipocalin NGAL in chronic *H. pylori*-induced gastritis and in gastric carcinoma. MMPs are the main degrading enzymes of extracellular matrix proteins and basement membranes and are therefore involved in tissue remodelling and repair as well as recruitment of inflammatory cells and angiogenesis in many physiological and pathological processes, including inflammation and malignancy. As introduction, a short overview is given in **chapter 1** on MMPs, TIMPs and lipocalins in general and their role in *H. pylori*-induced gastritis and gastric carcinoma in particular.

H. pylori gastritis is recognized as an important pathogenetic factor in peptic ulcer disease and gastric carcinogenesis. **Chapter 2** reports on the influence of *H. pylori* infection on gastric mucosal MMP-2 and -9 expression. In gastric mucosal biopsies

of individuals with or without *H. pylori* infection, the levels, isoforms and activity of MMP-2 and -9 were determined by quantitative gelatin-zymography, bioactivity assays (BIAs), enzyme-linked immunosorbent assays (ELISAs) and immunohistochemistry. In addition, the relation between gastric mucosal MMP-2 and -9 expression and severity of inflammation was assessed.

The influence of *H. pylori* eradication therapy on mucosal MMP-2 and MMP-9 levels is described in **chapter 3**. Gastric biopsies from patients with *H. pylori*-associated gastritis, that were treated with a combination regimen of acid inhibitory therapy and antibiotics in order to eradicate *H. pylori* and to reduce the risk for peptic ulcer disease, were evaluated for the levels, isoforms and activity of MMP-2 and MMP-9 by quantitative gelatin-zymography, bioactivity assays (BIAs) and enzyme-linked immunosorbent assays (ELISAs).

In a number of human cancers, enhanced expression of MMPs has been described in primary tumours associated with tumour progression and poor prognosis. **Chapter 4** describes the initial study of levels, isoforms, and activities of MMP-2 and MMP-9 in gastric carcinomas and corresponding normal mucosa, as assessed by quantitative gelatin–zymography. These parameters were correlated with a number of clinicopathological parameters including TNM stage and histological classifications according to Laurén and WHO. In addition, the prognostic significance of the MMP-2 and MMP-9 levels for the overall survival of the patients was evaluated.

The results of a more comprehensive study that was carried out to endorse the findings as described in chapter 4 are presented in **chapter 5**. The MMP analyses in the same group of patients were extended and compared with those obtained with a new and more recent group of gastric cancer patients. Furthermore, instead of quantitative gelatin-zymography, bioactivity assays (BIAs) and enzyme-linked immunosorbent assays (ELISAs) for MMP-2 and MMP-9 were used. Moreover, the prognostic value of MMP-2 and MMP-9 was compared with those of MMP-7 and MMP-8 and the study was expanded by determination of the inhibitors TIMP-1 and TIMP-2. In addition, because of the increasing age of the patients and the length of the follow-up, tumour-associated survival was evaluated.

Neutrophil gelatinase-associated lipocalin (NGAL) is a lipocalin that has initially been discovered in specific granules of human neutrophils and was later shown to be expressed also by certain epithelial cells, especially in inflamed or malignant tissues. A part of the NGAL is present as a complex with MMP-9. In **chapter 6,** an analysis of the presence of MMP-9-NGAL complexes in tissue extracts from gastric cancers and their relation with survival is presented. In the same cohort of patients as used in the study described in chapter 5, MMP-9-NGAL complexes were measured by zymography and by ELISA. The tumour levels of MMP-9-NGAL complex, MMP-9 and NGAL were evaluated for correlations with established clinicopathological parameters of

the gastric carcinoma patients and for their predictive value to patients' outcome. In addition, immunohistochemical analysis of serial paraffin-embedded tissue sections and immunofluorescence double staining were used to establish the cellular origin of MMP-9 and NGAL.

Single-nucleotide polymorphisms (SNPs) within MMP genes are thought to influence the expression of MMPs and/or even seem to be associated with the susceptibility for the development of malignancy. The clinical impact of MMP and TIMP gene polymorphisms in our cohort of gastric cancer patients is described in **chapter 7**. The genotype distribution and allele frequencies of SNPs of MMP-2, -7, -8 and -9 and TIMP-1 and -2 were studied. In order to get insight into the functional and clinical contribution of these MMP-related gene polymorphisms, the relationship between the distribution of these SNPs and the respective protein levels in tumour and adjacent normal tissue, as well as the relation of the SNPs with established clinicopathological parameters and tumour-related survival was assessed.

The different studies are finally compiled as a summarizing discussion in **chapter 8**, including a discussion on the potential role of MMP inhibition in gastric cancer.

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CHAPTER 2

Mucosal gelatinases MMP-2 and MMP-9 in *Helicobacter pylori*-associated gastritis

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Summary

Colonization of the gastric mucosa with *Helicobacter pylori* (*H. pylori*) leads to an acute gastritis, which will develop into a chronic gastric inflammatory reaction in the majority of infected individuals. This long-term *H. pylori*-associated gastritis is recognized as an important pathogenic factor in peptic ulcer disease and gastric carcinogenesis, disorders where tissue remodelling through matrix metalloproteinases (MMPs) is known to occur. The present study was performed to investigate whether the gastric mucosal MMP-2 and MMP-9 levels are affected by an *H. pylori* infection. The levels, isoform constitution and activity of MMP-2 and MMP-9 were determined by quantitative gelatin-zymography, bioactivity assay (BIA), Enzyme-Linked Immunosorbent Assay (ELISA) and immunohistochemistry in mucosal biopsies of the antrum and corpus originating from patients with an *H. pylori*- associated gastritis (n=45) and *H. pylori* negative control patients (n=27). *H. pylori* infection and gastritis was established by a combination of culture and/or histological identification, and confirmed by specific IgG *H. pylori* antibodies.

Patients with an *H. pylori* infection showed significantly elevated levels of all forms of MMP-9 (5- up to 70-fold, $P \leq 0.005$) in both antrum and corpus mucosa when compared with *H. pylori* negative controls, whereas the MMP-2 levels were almost identical. The increase of MMP-9 in the corpus mucosa of patients with antral gastritis only was less impressive (3- to 6-fold) and intermediate ($0.001 \leq P \leq 0.05$) to that of *H. pylori* negative patients and pangastritis patients. A highly significant correlation between quantitative gelatin-zymography, BIA and ELISA was observed for MMP-9 (R>0.81, $P<0.001$), which was less coherent for MMP-2. Semi-quantitative histology scores confirmed that both active as well as chronic inflammation were significantly ($P<0.001$) increased in antrum as well as in corpus mucosa of *H. pylori* infected patients compared with *H. pylori* negative patients. The increase of MMP-9, primarily expressed in phagocytic inflammatory cells, correlated significantly ($0.27 \leq R \leq 0.53$) with the severity of both the active as well as chronic inflammation in antrum ($P<0.05$) and particularly in corpus mucosa ($P<0.01$).

In conclusion, *H. pylori*-associated gastritis is characterized by a significant increase in the MMP-9 levels in both antrum and corpus mucosa, which is particularly present in phagocytes and correlates with the severity of the mucosal inflammation. In contrast, MMP-2 levels are almost unaltered when compared with *H. pylori* negative patients. This MMP-9 profile in the *H. pylori*-infected gastric mucosa is comparable to that seen in gastric ulceration and carcinomas.

Introduction

Helicobacter pylori (H. pylori) is a curved or spiral-shaped Gram-negative bacterium that lives in the mucus layer of the gastric epithelium and in metaplastic gastric epithelium of the oesophagus and duodenum [1-3]. Infection with *H. pylori* is the most common cause of gastritis, preceeded by colonization of the gastric mucosa [4]. This infection leads to an acute gastritis that, over the course of several weeks, will develop into a chronic inflammatory reaction of the mucosa [5]. Patients with longstanding *H. pylori*-associated chronic gastritis are predisposed for peptic ulcer disease as well as gastric carcinoma and lymphoma [6, 7]. In this sequence of events cells migrate through the gastric tissue that thereby undergoes constant remodelling.

Matrix metalloproteinases (MMPs) are thought to be key enzymes in these kinds of inflammatory, ulcerative, and malignant processes [8-12]. The (patho)physiologic degradation of basement membrane components and the extracellular matrix is executed by these secreted or transmembrane endo-proteinases, which share a zinc-containing catalytic domain required for their proteolytic activity. Currently, at least 17 MMP family members have been identified which can be divided into 4 major subgroups, based on their substrate preferences: i.e. collagenases, stromelysins, gelatinases and membrane-type MMPs. Most of these enzymes are secreted in a latent form and require extracellular activation. Their activity is regulated by the interaction with their antagonists, the tissue inhibitors of metalloproteinases (TIMPs), and through inhibition by α_2-macroglobulin [8, 11, 12]. Previous studies have shown that gastric ulceration, both in animal models and in humans, is accompanied by enhanced expression and levels of several MMPs within the mucosa [13-16]. In addition, gastric cancer is also reported to be characterized by an increase of MMPs [17-22]. The aim of the present study was to assess whether the levels of the gelatinases MMP-2 and MMP-9 in the gastric mucosa are affected already early in these pathophysiological processes, i.e. during an *H. pylori* infection, which has to our knowledge not been reported before.

Patients, materials and methods

Patients

Biopsy specimens at upper gastrointestinal endoscopy were obtained from 72 consecutive dyspeptic patients, between 21 and 80 years of age (mean age: 51.8±1.9 years), consisting of 42 males and 30 females. Patients who had recently used proton-pump inhibitors, corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), bismuth compounds, sucralfate, or antibiotics were excluded. For histological examination, 2

biopsies were taken from the antrum, 3-5 cm proximal to the pylorus, and 2 from the corpus, 5 cm above the junction between antrum and corpus. These specimens were examined by an experienced pathologist according to the guidelines of the revised Sydney system, which provides a semi-quantitative grading of histological parameters (0 = normal, 1 = mild, 2 = moderate, 3 = marked) [23]. One biopsy was taken from the antrum for *H. pylori* culture and processed as described previously [24]. In 27 cases, the stomach was *H. pylori* negative, which is defined as normal, while 31 patients had a pangastritis. In these cases both antrum and corpus showed histological signs of inflammation. In the other 14 cases, only the antrum was inflamed. All cases of gastritis were caused by *H. pylori*. The presence of these bacteria was assessed by a culture and/or histological identification, and confirmed by specific IgG *H. pylori* antibodies. Two biopsies of antrum and corpus were used for the determination of MMP-2 and MMP-9.

Tissue extraction and protein concentration

Homogenates were made by adding 100 µl PBST (0.05% Tween®20 in phosphate buffered saline) per mg biopsy material and homogenizing on ice in a Potter S (B. Braun). The protein concentration in the supernatant was determined by the Lowry method [25].

Gelatin-zymography

The presence of active and pro forms of the matrix metalloproteinases were assessed by gelatin-zymography, as previously described [18]. Ten per cent polyacrylamide gels were casted in a Mini-Protean® II Dual Slab Cell (Biorad). These gels contained 1.5M Tris buffer (pH 8.8), 0.2% gelatin, 0.1% sodium dodecyl sulphate, 0.07% ammonium persulphate and 0.07% tetramethylenediamine. First sample volumes were adjusted to obtain an equal protein content of 5 µg per sample. Two amounts (6.1 and 12.2 µg protein) of an internal standard preparation, i.e. a homogenate of a colonic carcinoma containing both MMP-2 and MMP-9, were included on each gel for correction of intergel variation and as reference for the expression in arbitrary units. After electrophoresis the gels were incubated overnight at 37°C, stained with Amido Black (0.1% amido black, 30% methanol and 10% acetic acid), and destained in a solution containing 30% methanol and 10% acetic acid. Subsequently the gels were dried between sheets of cellophane. Finally the degree of gelatin digestion was quantified by making a digital photo with a CCD Imaging System (Appligene), scanned in Aldus Photostyler 2.0 (Aldus Corporation) and analysed with Imagequant (Molecular Dynamics), using the peakfinder-mode. The gelatin digestion was reflected as a peak and the MMP levels were calculated referring to the internal standard preparations, of which the peak-height correlated highly significant with the included concentration

(R=0.99, $P < 0.001$). The MMPs were analysed for the pro, active and total MMP levels, the latter defined as the sum of the two isoforms, and expressed as Arbitrary Units per 5 µg protein.

Bioactivity assay

Latent (activatable) and active MMP were also measured using a newly developed immunocapture colorimetric activity assay (BIA) [26, 27]. Briefly, a polyclonal anti-MMP-2 or monoclonal anti-MMP-9 antibody (TNO-PG) was used as catching antibody to capture MMP-2 or MMP-9 from appropriate dilutions of the tissue homogenates, respectively 1:4, and 1:20, by overnight incubation at 4°C. Active MMP was determined directly, whereas latent MMP was activated by incubation with 0.5 mM APMA (*p*-aminophenylmercuric acetate) for 0.5 and 2 hr at 37°C for MMP-2 and MMP-9, respectively. After washing MMP activity was assessed by adding 750 ng modified MMP-activatable pro-urokinase (Ukcol) and 0.6 mM of its chromogenic substrate S-2444 (pyro-Glu-Gly-Arg-*p*-nitroanilide; Chromogenix, Sweden) in assay buffer and incubating at 37°C. Reactions were performed in 96-well flat-bottomed microtitre plates, and a Titertek Multiskan photometer was used to follow the absorbance kinetics at 405 nm. Results were expressed as MMP activity Units per mg protein, with Units defined as $(\Delta A_{405}/hr^2)*10$.

ELISAs

The total amount of MMP-2 and MMP-9 protein was determined by sandwich-ELISAs [27]. In brief, the same catching antibodies were used as for the immunocapture activity assays and appropriate dilutions of tissue homogenates, respectively 1:6.7 and 1:5, were incubated overnight at 4°C. Immunodetection of MMP-9 was performed with biotinylated rabbit anti-MMP-9 and for MMP-2 using rabbit anti-MMP-2 (TNO-PG) followed by biotinylated goat anti-rabbit-IgG. After incubation with avidin/horseradish-peroxidase the chromogenic substrate 3,3',5,5'-tetramethyl benzidine and H_2O_2 were added and the reaction was stopped with H_2SO_4 and read at 405 nm. The amount of MMP was calculated from the parallel standard curves and expressed in ng MMP per mg protein.

Immunohistochemistry

MMP-2 and MMP-9 were localized by routine indirect peroxidase-labelled antibody immunohistochemistry. Briefly, 4 µm paraffin sections were sequentially treated with 0.005% proteinase K (Boehringer Mannheim, Germany) in Tris-buffered saline (TBS) to retrieve hidden antigens and with 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. After permeabilization in 0.5% Triton X-100 in 0.1% sodium citrate the sections were rinsed in TBS and 5% normal goat serum was applied for 20 minutes

to block non-specific binding. Excess serum was drained off, and sections were incubated overnight at 4°C with rabbit anti-MMP-2 or anti-MMP-9 polyclonal antibodies, appropriately diluted in TBS containing 0.5% BSA. The sections were subsequently incubated with biotinylated goat anti-rabbit IgG and peroxidase-labelled streptavidin for 45 minutes each. Sections were stained by incubation in 0.1 M acetate buffer (pH 5.2) containing 0.03% 3-amino-9-ethylcarbazole and 0.03% H_2O_2 for 10 minutes, resulting in a red staining product. Finally, sections were counterstained in Mayer's haematoxylin and mounted in Aquamount™.

Statistical analysis

Group means are given as mean ± s.e.m. Differences between groups were evaluated for significance using the Kruskal-Wallis and Mann-Whitney *U* tests or the Wilcoxon Signed-Ranks test. The correlations between zymography, immunocapture activity assay, and ELISA were assessed by the Pearson correlation procedure (SPSS for Windows 7.0 statistical package, SPSS Inc., Chicago, Illinois, U.S.A.). Differences were considered significant when $P \leq 0.05$.

Results

Patients with an *H. pylori* infection were found to have a significantly higher level of total MMP-9, as assessed by zymography, in both antrum [216±50 (n=45) *vs*. 4.8±1.5 (n=27), P<0.001] and corpus mucosa [100±20 (n=45) *vs*. 4.2±1.2 (n=27), P<0.001] compared

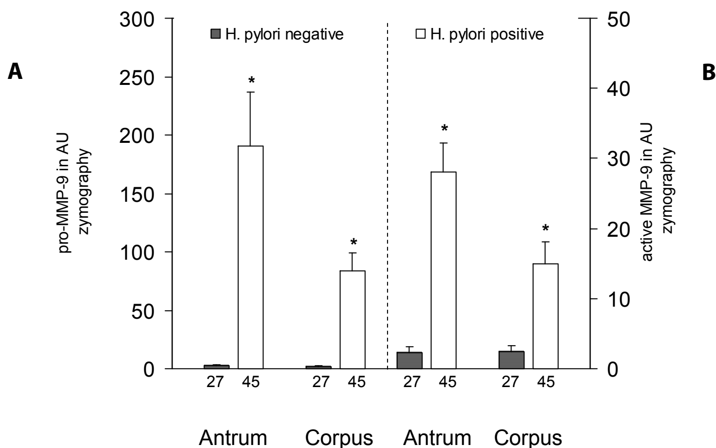

Figure 1. Mean levels of pro (A) and active (B) MMP-9 ± s.e.m. in antrum and corpus mucosa originating from subjects with or without an *H. pylori* infection, as assessed by gelatin-zymography. The number of patients is indicated. * P < 0.001

with patients who where *H. pylori* negative. The same was true for both zymographic MMP-9 isoforms, i.e. pro and active MMP-9, which were also significantly increased in both antrum and corpus mucosa (Figure 1). These results were further substantiated by the BIA and ELISA for MMP-9. Latent MMP-9 as well as the total amount of MMP-9 protein were also found to be approximately 6- to 30-fold enhanced in both antrum and corpus mucosa of *H. pylori* positive versus *H. pylori* negative patients (Figure 2). Active MMP-9, as assessed by the BIA, was found to be similarly enhanced in both antrum

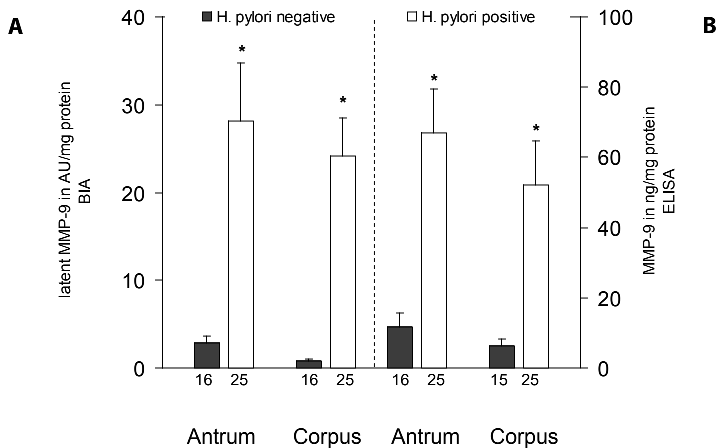

Figure 2. Mean levels of latent or activatable (A) and total amount protein (B) MMP-9 ± s.e.m. in antrum and corpus mucosa originating from subjects with or without an *H. pylori* infection, as determined by BIA and ELISA, respectively. The number of patients is indicated. * $P \leq 0.005$

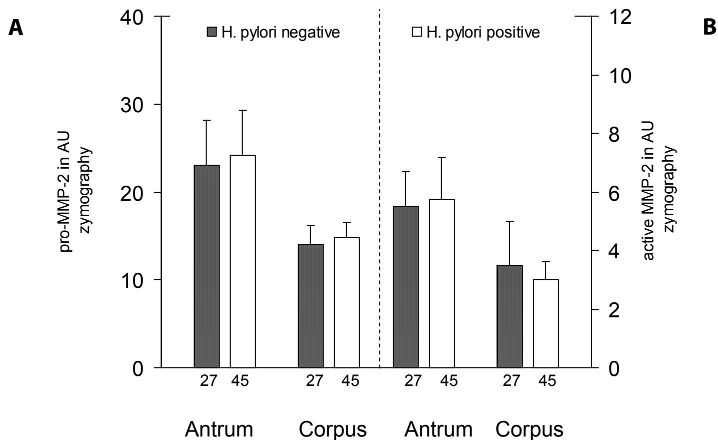

Figure 3. Mean levels of pro (A) and active (B) MMP-2 ± s.e.m. in antrum and corpus mucosa originating from subjects with or without an *H. pylori* infection, as assessed by gelatin-zymography. The number of patients is indicated. Differences between *H. pylori* positive and *H. pylori* negative patients were not significant.

[9.6±2.0 (n=25) *vs.* 1.1±0.4 (n=16), $P<0.001$] and corpus mucosa [8.0±2.2 (n=25) *vs.* 0.6±0.2 (n=16), $P<0.001$] compared with patients who where *H. pylori* negative. Highly significant correlations between the different detection assays, i.e. zymography, BIA and ELISA, for MMP-9 were found in both tissue types ($0.81<R<0.91$, $P<0.001$).

In contrast, the total MMP-2 levels in the zymographic analyses were found to be almost identical in the two patient-groups, in antrum [30±6.6 (n=45) *vs.* 29±6.3 (n=27), NS] as well as in corpus mucosa [19±1.9 (n=45) *vs.* 18±2.9 (n=27), NS]. The levels of the pro and active isoform of MMP-2 were also found to be highly similar in the gastric mucosa of *H. pylori* positive and negative patients (Figure 3). These results were confirmed by the BIA and ELISA for MMP-2 in these tissues. Latent MMP-2 in the antrum [2.1±0.5 (n=23) *vs.* 2.8±0.7 (n=15), NS] and corpus mucosa [2.4±0.5 (n=25) *vs.* 2.8±0.5 (n=15), NS] of patients with an *H. pylori* infection were almost identical to controls. The ELISA for MMP-2 also failed to show significant differences between *H. pylori* positive and negative patients, i.e. 6.7±1.3 (n=25) *vs.* 7.7±2.1 (n=15) in antrum and 6.4±1.3 (n=25) *vs.* 4.5±0.7 (n=17) in corpus mucosa. Active MMP-2 by the BIA was not assessed because of the absence of differences in the other determinations. Overall, the differ-

Table 1 - Mean values of MMP-2 and -9 in antrum and corpus mucosa, as assessed by gelatin-zymography and given in Arbitrary Units (AU) ± s.e.m. Total MMP is defined as the sum of pro and active MMP. Normal mucosa is defined as *H. pylori* negative.

Antrum		Normal Mucosa (n=27)	Antral Gastritis (n=14)	Pangastritis (n=31)
MMP-2	Pro	23 ± 5.1	19 ± 3.4	27 ± 7.0
	Active	5.5 ± 1.2	2.7 ± 0.7	7.0 ± 3.0
	Total	29 ± 6.3	21 ± 3.5	34 ± 9.4
MMP-9	Pro	2.6 ± 0.7	108 ± 34 °[1]	224 ± 64 •[1]
	Active	2.3 ± 0.8	20 ± 4.9 °[1]	31 ± 6.0 •[1]
	Total	4.8 ± 1.5	127 ± 38 °[1]	256 ± 69 •[1]

Corpus		Normal Mucosa (n=27)	Antral Gastritis (n=14)	Pangastritis (n=31)
MMP-2	Pro	14 ± 2.2	13 ± 1.9	17 ± 2.2
	Active	3.5 ± 1.5	2.0 ± 0.9	3.5 ± 0.7
	Total	18 ± 2.9	15 ± 2.0	20 ± 2.6
MMP-9	Pro	1.8 ± 0.5	12 ± 3.7 °[2]	119 ± 22 •[1]◊[1]
	Active	2.4 ± 0.9	6.0 ± 2.7	18 ± 4.3 •[1]◊[2]
	Total	4.2 ± 1.2	18 ± 5.7 °[3]	137 ± 26 •[1]◊[1]

The following significances are based on the differences between the groups.
• = pangastritis compared with normal mucosa
° = antral gastritis compared with normal mucosa
◊ = pangastritis compared with antral gastritis
[1] $P \leq 0.001$, [2] $P \leq 0.01$, [3] $P \leq 0.05$

ent detection assays for MMP-2 were found to be less coherent than for MMP-9, with the best correlation found between the BIA and the ELISA (0.46<R<0.67, $P<0.005$).

Both *H. pylori* infected groups, i.e. patients with antral gastritis only or those with pangastritis, showed remarkably higher levels of MMP-9 in both antrum and corpus compared to control mucosa, exemplified by the zymographic results (Table 1). Considering the *H. pylori* positives separately, the corpus mucosa of patients with pangastritis showed significantly higher pro, active, and total MMP-9 levels than that of patients with antral gastritis only, whereas in the antrum mucosa a similar trend was observed, without reaching statistical significance. In addition, patients with an antral gastritis only were found to have significantly elevated levels of the MMP-9 isoforms ($0.002<P<0.05$) within the antrum mucosa when compared with their corresponding corpus mucosa. In the patients with pangastritis and the *H. pylori* negative patients the antrum only showed somewhat higher MMP-9 levels, although not statistically significant, when compared with their corpus mucosa.

The antrum of *H. pylori*-infected patients showed significantly more active inflammation [1.3±0.1 (n=44) *vs.* 0 (n=27), $P<0.001$] as well as chronic inflammation [1.5±0.1 (n=44) *vs.* 0.6±0.1 (n=27), $P<0.001$] compared with the *H. pylori* negative patients. A similar effect was seen in the corpus mucosa of *H. pylori*-infected patients for both active inflammation [0.7±0.1 (n=42) *vs.* 0 (n=26), $P<0.001$] and chronic inflammation [1.2±0.1 (n=42) *vs.* 0.5±0.1 (n=26), $P<0.001$]. Compared with *H. pylori* negative patients, both *H. pylori*-infected groups showed significantly more active as well as chronic inflammation in the antrum (Table 2). A similar trend was found in the corpus mucosa for active inflammation, whereas chronic inflammation was only significantly

Table 2 - Mean values ± s.e.m. of active, i.e. presence of neutrophilic polymorphonuclear leukocytes, and chronic, i.e. cellular infiltrate consisting of lymphocytes, plasma cells, monocytes, mast cells and eosinophils, inflammation in antrum and corpus mucosa [23]. Normal mucosa is defined as *H. pylori* negative.

Inflammation Antrum	Normal Mucosa (n=27)	Antral Gastritis (n=14)	Pangastritis (n=30)
Active	0	1.2 ± 0.1 °[1]	1.3 ± 0.1 •[1]
Chronic	0.6 ± 0.1	1.4 ± 0.1 °[1]	1.5 ± 0.1 •[1]

Inflammation Corpus	Normal Mucosa (n=26)	Antral Gastritis (n=14)	Pangastritis (n=28)
Active	0	0.1 ± 0.1 °[3]	1.0 ± 0.1 •[1]◊[1]
Chronic	0.5 ± 0.1	0.7 ± 0.2	1.4 ± 0.1 •[1]◊[2]

The following significances are based on the differences between the groups.
• = pangastritis compared with normal mucosa
° = antral gastritis compared with normal mucosa
◊ = pangastritis compared with antral gastritis
[1] $P \leq 0.001$, [2] $P \leq 0.01$, [3] $P \leq 0.05$

higher in patients with pangastritis. Regarding the two *H. pylori*-infected patient-groups separately, the corpus of patients with pangastritis showed significantly more active as well as chronic inflammation than that of patients with antral gastritis only, in contrast to the corresponding antrum in which a similar intensity of active and chronic inflammation was found. As expected, the antrum of patients with antral gastritis only showed significantly higher scores of active (P<0.001) and chronic inflammation (P<0.01) compared with their corresponding corpus mucosa. Within the *H. pylori* negatives only minimal chronic inflammation was detected in both antrum and corpus mucosa.

Immunohistochemically MMP-9 was predominantly observed in inflammatory and stromal cells, i.e. neutrophilic granulocytes, macrophages, and (myo)fibroblasts, and in zymogen producing chief cells of corpus mucosa (data not shown). Faint MMP-2 immunoreactivity was predominantly observed in inflammatory cells as well, but not in chief cells.

In the overall patient-group, i.e. *H. pylori* positives and negatives together, a significant correlation was found between the zymographically determined total MMP-9 levels and the active as well as chronic inflammation in both antrum [respectively, R=0.27, P<0.05 and R=0.33, P<0.01 (n=71)] and corpus mucosa [respectively, R=0.53 and R=0.45, both P<0.001 (n=68)]. In the corpus mucosa of patients with an *H. pylori* gastritis both the active and chronic inflammation correlated significantly with these MMP-9 levels [respectively, R=0.38 and R=0.36, both P<0.05 (n=42)], in contrast to the antrum where no correlation was found.

Discussion

Previous studies revealed an increased expression of MMPs during gastric ulceration [13-16] and in carcinomas of the stomach [17-22]. *H. pylori*-associated chronic gastritis is known to be able to evolve in peptic ulcer disease or gastric cancer in some patients. Therefore, we assessed the expression of the gelatinases MMP-2 and MMP-9 in gastric mucosal biopsies from patients with a *H. pylori* gastritis. We found *H. pylori* positive patients to have significantly elevated levels of MMP-9 in antrum and corpus mucosa when compared with *H. pylori* negative patients, whereas the MMP-2 levels were highly similar. The increase in MMP-9 was very consistent and irrespective of the detection technique used, i.e. gelatin-zymography, BIA or ELISA. Moreover, not only the total mucosal MMP-9 protein level was enhanced due to the *H. pylori* infection, also the two isoforms latent (pro) and active MMP-9 were similarly increased. With regard to MMP-2 no changes were found neither in the total mucosal protein level nor in the isoform composition.

The significantly elevated levels of mucosal MMP-9 in patients with an *H. pylori*-associated gastritis was found to be primarily localized in the infiltrating inflammatory cells such as macrophages, neutrophils and also in some (myo)fibroblasts. The presence and activation of these cells is most probably caused by the locally produced mucosal substances like cytokines, e.g. TNF-α and IL-8, which have been reported to be increased in *H. pylori*-induced chronic gastritis [28-31]. The finding of a significant difference in the corpus MMP-9 levels between *H. pylori* negative controls and patients with only an antral gastritis is remarkable. This might be due to both the presence of some minimal active inflammation and to proinflammatory alterations occurring in the corpus, induced by mediators such as cytokines and cytotoxins, originating from the infected and inflamed adjacent antrum, thereby creating a kind of paracrine stimulation.

The antrum of patients with an antral gastritis only showed significantly higher levels of MMP-9 compared with their corresponding corpus. The antrum of *H. pylori* negative patients and that of patients with a pangastritis, however, contained similar MMP levels compared with their corpus mucosa. Therefore, one might conclude that there is at least no intrinsic difference between antrum and corpus mucosa, regarding the MMP levels. The inflammatory reaction in the corpus mucosa of patients with an antral gastritis only was found to be less intense compared with the antrum mucosa as illustrated by the smaller amount of inflammatory cell infiltrate. This is probably related to the slow pyloro-cardial progression of gastritis as a consequence of a less dense *H. pylori* colonization of the corpus due to local acid production [32]. The differences in MMP-9 levels found between the antrum and corpus mucosa of antral gastritis patients are thus most probably caused by the larger amount of MMP producing and secreting cells present within the antrum mucosa. In addition, within the antrum of all the patients a fairly good correlation was found between the MMP-9 level and the severity of both the active and chronic inflammation, which was even better in the corpus mucosa. This latter observation might be explained by the fact that the corpus mucosa of patients with an antral gastritis is intermediately inflamed, when compared with *H. pylori* negatives and patients with a pangastritis, causing a more gradual increase of inflammation and accompanying MMP secreting inflammatory cells.

Gastric ulceration is known to be accompanied by an enhanced expression of several metalloproteinases within the mucosal lesions, but gelatinases have only been assessed in animal models [13-16]. Interestingly also in the acetic acid-induced ulcers in rats only MMP-9 was found to be impressively increased whereas MMP-2 expression was hardly affected [13, 15]. These findings are highly similar to our observations in *H. pylori*-associated gastritis. In contrast, studies in patients with gastric cancer, including one by our group using similar techniques, consistently revealed that both MMP-2

and -9 levels are significantly elevated within the malignant tissue [18-20]. Apparently the premalignant inflammatory and ulcerative lesions are accompanied by the induction of only MMP-9, whereas the end-stage of the spectrum, i.e. gastric cancer, is characterized by a general upregulation of both gelatinases. Part of this difference might be explained by the fact that MMP-9 is an inducible matrix metalloproteinase in contrast to MMP-2, which is expressed constitutively [8, 11]. Particularly with regard to the *H. pylori* infection it has been reported that the inflammation is associated with an increase of mucosal cytokines [28-31], which are able to enhance the MMP-9 production. Furthermore, the divergence in MMP-2 and MMP-9 induction is probably also related to the differences in predominant cellular origin of these gelatinases. MMP-2 is expressed amongst others by stromal fibroblasts and epithelial cells [11, 33, 34], whereas MMP-9 is particularly secreted by cells of the phagocytic lineage [11, 35-38], which fits well with our immunohistochemical results in the *H. pylori*-infected gastric mucosa. Further prospective studies in patients with an *H. pylori*-associated chronic gastritis with a long follow-up might help to identify whether those patients with a relatively high MMP-2 level in the mucosa are the ones with the strongest predisposition for gastric carcinoma.

Another aspect of the high MMP-9 levels in *H. pylori* gastritis might be the relation with wound healing. Acute wounds and impaired healing, i.e. chronic persistent wounds, have been shown to be accompanied by a high expression of MMP-9 [39-41]. Therefore, it is likely that the excessive MMP-9 expression as found in the *H. pylori*-infected mucosa, may prevent spontaneous healing. Previous studies by our group indicate that successful eradication of *H. pylori* leads to improvement and normalization of the active and chronic inflammatory reaction in the stomach, which is accompanied by a reversal of alterations in other mucosal parameters, e.g. plasminogen activators and superoxide dimutases [42, 43]. Similar studies will have to elucidate whether the MMP-9 levels will also be normalized by eradication of the *H. pylori* infection.

In conclusion, *H. pylori*-associated gastritis is characterized by a significant increase of MMP-9 in both antrum and corpus mucosa of the stomach, with no changes in MMP-2, compared with *H. pylori* negative patients. Moreover, the increase of the gastric mucosal MMP-9 level is significantly correlated with the severity of both the active as well as the chronic inflammation. Future studies will elucidate the clinical relevance of these findings by evaluating the effect of therapy and the association with gastric carcinogenesis.

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CHAPTER 3

Eradication of *Helicobacter pylori* infection favourably affects altered gastric mucosal MMP-9 levels

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Summary

Background

Helicobacter pylori gastritis is recognized as an important pathogenetic factor in peptic ulcer disease and gastric carcinogenesis, and is accompanied by strongly enhanced gastric mucosal MMP-9 levels.

Aim

This study was performed to investigate whether *Helicobacter pylori*-affected gastric mucosal MMP-2 and MMP-9 levels are reversible by successful treatment of the infection.

Patients and methods

Fifty-eight patients with *H. pylori*-associated gastritis were treated with a combination regimen of acid inhibitory therapy and antibiotics for 14 days. The levels and isoforms of MMP-2 and MMP-9 were measured by semi-quantitative gelatin-zymography, bioactivity assay (BIA) and enzyme-linked immunosorbent assay (ELISA) in gastric mucosal biopsy homogenates.

Results

Latent, active and total MMP-9 levels decreased consistently and significantly by successful *H. pylori* eradication, in antrum as well as corpus mucosa, compared with those prior to treatment, irrespective of the therapy regimen used. The elevated levels remained unchanged, however, when treatment failed. MMP-2 levels did not show major alterations after *H. pylori* therapy.

Conclusions

Elevated MMP-9 levels in *H. pylori*-infected gastric mucosa are reversible by eradication of the infection. No major changes in mucosal MMP-2 levels were observed by *H. pylori* eradication.

Introduction

Helicobacter pylori (*H. pylori*) is a curved or spiral-shaped Gram-negative bacterium that lives in the mucus layer of the gastric epithelium and also in metaplastic gastric epithelium of the esophagus or duodenum [1-3]. Infection with *H. pylori* is the most common cause of gastritis [4] and is preceded by colonization of the gastric mucosa. This infection leads to an acute gastritis that, over the course of several weeks, develops into a chronic inflammatory reaction of the mucosa [5]. Patients with long-term *H. pylori*-associated chronic gastritis are predisposed for peptic ulcer disease as well as gastric carcinoma and lymphoma [6, 7]. Matrix metalloproteinases (MMPs) are believed to play an important role in inflammation and carcinogenesis, amongst others, via the degradation and remodeling of extracellular matrix and basal membranes [8, 9]. MMPs are secreted or transmembrane endo-proteinases that share a zinc-containing catalytic domain, which is required for proteolytic activity. MMPs can degrade at least one component of the extracellular matrix. Currently, at least 25 family members have been identified which can be divided in four major subgroups, based on substrate specificity, amino acid similarity, and identifiable sequence modules: collagenases, stromelysins, gelatinases, and membrane-type MMPs. The proteins are secreted in a latent form and require extracellular activation. When activated, the enzymes are susceptible to inhibition by α2-Macroglobulin and by their antagonists, the Tissue Inhibitors of MetalloProteinases (TIMPs), by forming a complex with the (active) enzyme. This complex formation is believed to be a major regulatory mechanism [9, 10].

The gelatinases include MMP-2 or gelatinase-A, a 72 kDa proteinase, and MMP-9 or gelatinase-B, a 92 kDa proteinase, which specifically can degrade basement membrane type IV collagen, as well as gelatin, collagen type I, V, VII , X, elastin, laminin and fibronectin [11, 12]. MMP-2, an ubiquitous enzyme in normal adult tissue, is predominantly produced by stromal cells, whereas MMP-9 is predominantly produced by inflammatory cells, especially the polymorphonuclear leucocytes [9, 11, 13, 14].

In gastric biopsies from *H. pylori*-infected individuals enhanced levels of MMP-2 and MMP-9 have been described, whereas TIMP-1 and TIMP-2 levels were unaltered [15]. We previously demonstrated increased MMP-9 levels in antrum and corpus mucosa of individuals with *H. pylori*-associated gastritis, with almost unchanged MMP-2 levels, compared to *H. pylori* negative patients [16]. Furthermore, we recently reconfirmed our observation of enhanced MMP-2 and MMP-9 levels in gastric carcinoma tissues and found a consistent independent association between MMP-2 levels and patient survival [17]. As *H. pylori* gastritis is associated with gastric malignancy and *H. pylori* gastritis and gastric carcinomas are accompanied by alterations in the MMP levels we decided to investigate whether gastric mucosal MMP-2 and MMP-9 levels in *H. pylori*-induced gastritis are affected by successful eradication of the infection.

Patients, materials and methods

Patients

Biopsy specimens were collected at upper gastrointestinal endoscopy from *H. pylori* positive patients between 22 and 75 years presenting with dyspeptic complaints, as described previously [18, 19]. Patients who had recently used proton-pump inhibitors, corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), bismuth compounds, sucralfate, or antibiotics were excluded. Use of low dose H_2-receptor antagonists was not considered to be a reason for exclusion. For histological examination, 2 biopsies were taken from the antrum, 3-5 cm proximal to the pylorus, and 2 from the corpus, 5 cm above the junction between antrum and corpus. These specimens were examined by an experienced pathologist according to the guidelines of the revised Sydney system, which provides semi-quantitative grading of histological parameters (0=normal, 1=mild, 2=moderate, 3=marked) [20]. One biopsy was taken from the antrum for *H. pylori* culture and processed as described previously [21]. The presence of *H. pylori* was assessed by a culture and/or histological identification, and confirmed by specific IgG *H. pylori* antibodies. From 58 of the 63 patients included in the original study there was still biopsy material of antrum and/or corpus available for the present study to determine the MMP-2 and MMP-9 concentrations. 33 of these patients had an antral gastritis, 23 patients had a pangastritis, data of two patients were missing.

All 58 patients were treated with a combination regimen of acid-suppression and antibiotics [omeprazole 20 mg bid in 26 patients, 16 male, 10 female, mean age 53 (range 22-75) or ranitidine 150 mg bid or 300 mg qid in 32 patients, 26 male, 6 female, mean age 46 (range 22-74) with clarithromycin 500 mg tid and metronidazole 500 mg tid for 14 days, the latter only in 50% of the omeprazole patients]. These combinations are further referred to as omeprazole and ranitidine, respectively. Successful treatment was defined as negative culture and negative histology eight weeks after the end of therapy. Four patients treated with omeprazole (double) therapy kept gastric complaints and were allowed to continue omeprazole use. They were found to be still *H. pylori* positive after therapy [3 male, 1 female, mean age 39.5 (range 24-58)].

Tissue extraction and protein concentration

Homogenates were made by adding 100 µl PBST (0.05% Tween®20 in phosphate buffered saline) per mg biopsy material and homogenizing on ice in a Potter S (B. Braun) [21]. The protein concentration in the supernatant was determined by the Lowry method [22].

Gelatin-zymography

The presence of active and pro forms of the matrix metalloproteinases were assessed by gelatin-zymography, as previously described [23, 24]. Ten percent polyacrylamide gels were casted in a Mini-Protean® II Dual Slab Cell (Biorad). These gels contained 1.5M Tris buffer (pH 8.8), 0.2% gelatin, 0.1% sodium dodecyl sulphate, 0.07% ammonium persulphate and 0.07% tetramethylene-diamine. First sample volumes were adjusted to obtain an equal protein content of 5 µg per sample. Two amounts (6.1 and 12.2 µg protein) of an internal standard preparation, i.e. a homogenate of a colonic carcinoma containing both MMP-2 and MMP-9, were included on each gel for correction of intergel variation and as reference for the expression in arbitrary units. After electrophoresis the gels were incubated overnight at 37°C, stained with Amido Black (0.1% amido black, 30% methanol and 10% acetic acid), and destained in a solution containing 30% methanol and 10% acetic acid. Subsequently the gels were dried between sheets of cellophane. Finally the degree of gelatin digestion was quantified by making a digital photo with a CCD Imaging System (Appligene), scanned in Aldus Photostyler 2.0 (Aldus Corporation) and analysed with Imagequant (Molecular Dynamics), using the peakfinder-mode. The gelatin digestion was reflected as a peak and the MMP levels were calculated referring to the internal standard preparations, of which the peak-height correlated highly significant with the included concentration ($r=0.99$, $p< 0.001$). The MMPs were analysed for the pro, active and total MMP levels, the latter defined as the sum of the two isoforms, and expressed as Arbitrary Units per 5 µg protein.

Bioactivity assay

Latent (activatable) and active MMP were also measured using a newly developed immunocapture colorimetric activity assay [17, 24]. Briefly, a polyclonal anti-MMP-2 or monoclonal anti-MMP-9 antibody (TNO-QLBR) was used as catching antibody to capture MMP-2 or MMP-9 from appropriate dilutions of the tissue homogenates, respectively 1:4 and 1:20, by overnight incubation at 4°C. Active MMP was determined directly, whereas latent MMP was activated by incubation with 0.5 mM *p*-aminophenylmercuric acetate for 0.5 and 2 hr at 37°C for MMP-2 and MMP-9, respectively. After washing MMP activity was assessed by adding 750 ng modified MMP-activatable pro-urokinase (Ukcol) and 0.6 mM of its chromogenic substrate S-2444 (pyro-Glu-Gly-Arg-*p*-nitroanilide; Chromogenix, Sweden) in assay buffer and incubating at 37°C. Reactions were performed in 96-well flat-bottomed microtitre plates, and a multichannel photometer was used to follow the absorbance kinetics at 405 nm. Results were expressed as MMP activity Units per mg protein, with Units defined as $(\Delta A_{405}/hr^2)*10$.

ELISAs

MMP-2 and MMP-9 protein levels were measured by our highly specific ELISAs, which detected the grand total of pro-enzyme, active- and inhibitor-complexed forms of the respective MMP, as previously described [17, 24]. In brief; the same catching antibodies were used as for the bio activity assays and appropriate dilutions of tissue homogenates, respectively 1:6.7 and 1:5, were incubated overnight at 4°C. Immunodetection of MMP-9 was performed with biotinylated rabbit anti-MMP-9 and for MMP-2 using rabbit anti-MMP-2 (TNO-PG) followed by biotinylated goat anti-rabbit-IgG. After incubation with avidin/horseradish-peroxidase the chromogenic substrate 3,3',5,5'-tetramethyl benzidine and H_2O_2 were added and the reaction was stopped with H_2SO_4 and read at 405 nm. The amount of MMP was calculated from the parallel standard curves and expressed in ng MMP per mg protein.

Statistical analysis

The ELISA, zymography and BIA results are given as mean ± s.e.m. Differences between groups were evaluated for significance using the Kruskal-Wallis and Mann-Whitney *U* tests or the Wilcoxon Signed-Ranks test. The correlations between zymography, BIA and ELISA were assessed by the Pearson correlation procedure (SPSS for Windows 11.0 statistical package, SPSS Inc., Chicago, Illinois, U.S.A.). Differences were considered significant when $P \leq 0.05$.

Results

ELISA

Overall MMP-9 levels measured by ELISA showed a significant decrease after successful therapy in both antral and corpus mucosa (Table 1). No relevant changes in MMP-9 levels were found in the four patients with persistent *H. pylori* infection, either in antrum or in corpus. The changes in the gastric MMP-9 levels were similar in the ranitidine and omeprazole treatment groups (data not shown). In addition, the levels

Table 1 - Overall MMP-2 and -9 levels in gastric mucosa biopsy specimens of *H. pylori* positive patients before and after treatment as measured by ELISA

Biopsy site	Therapy result	MMP-2			MMP-9		
		Before	After	*P*-value	Before	After	*P*-value
Antrum	Successful, *n* = 49/53	12.2 ± 0.7	10.0 ± 0.8	0.025	15.1 ± 1.7	2.2 ± 0.4	< 0.001
	Unsuccessful, *n* = 4	15.4 ± 2.7	8.3 ± 1.1	NA	9.4 ± 2.7	12.0 ± 6.8	NA
Corpus	Successful, *n* = 52/53	8.0 ± 0.6	7.1 ± 0.7	NS	5.2 ± 0.8	1.5 ± 0.4	< 0.001
	Unsuccessful, *n* = 4	7.4 ± 1.0	7.9 ± 1.8	NA	6.3 ± 2.6	9.5 ± 7.3	NA

Levels are expressed in ng / mg protein; NA : not applicable; NS : not significant

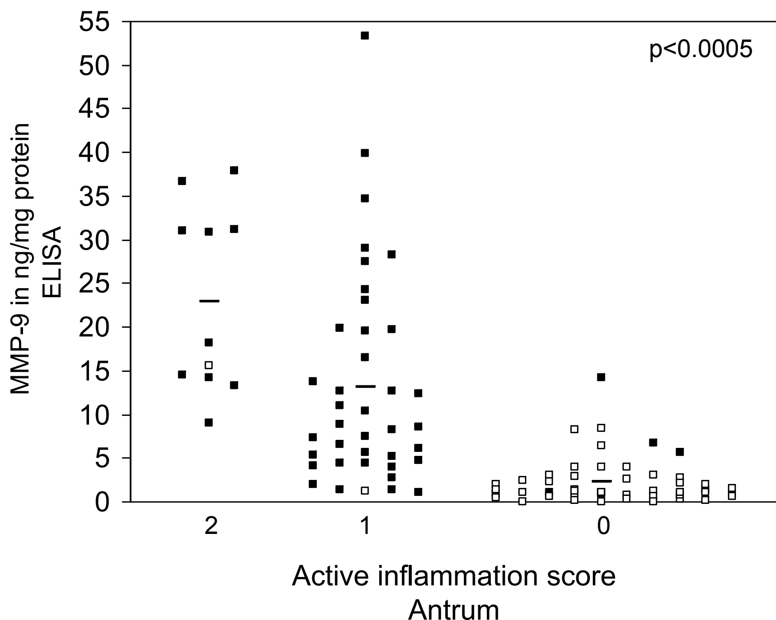

Figure 1. Scatter plot of the MMP-9 levels in the antrum, as measured by ELISA, in relation to active inflammation, as scored by immunohistological evaluation, combined of biopsies from before (■) and after (□) treatment of the *H. pylori* infection. Means per inflammation score group, as indicated by the horizontal bar, were 22.9 ± 3.2 (score 2, *n*=11), 13.1 ± 1.9 (score 1, *n*=39) and 2.3 ± 0.4 ng MMP-9/mg protein (score 0, *n*=49). Statistical significance of the association according to the Kruskall Wallis test *P* <0.0005.

of MMP-9 in the gastric mucosa were found to be strongly related to the severity of the active inflammation. This was particularly noticeable in the corpus mucosa where the MMP-9 level in the patients with a pangastritis (7.37 ± 1.46 ng/mg protein, *n*=22) was significantly higher (*P*<0.02) compared to those with an antral gastritis (3.68 ± 0.84, *n*=27). After eradication of *H. pylori* these levels were found to be significantly decreased (*P*<0.01) in both groups but no longer significantly different between both groups (respectively, 2.56 ± 0.87 and 0.73 ± 0.19). Furthermore, the MMP-9 levels in the antrum were also found to be significantly correlated with the severity of the inflammation, as illustrated by the stepwise decrease in the MMP-9 level in accordance with the inflammation score of the combined pre- and post-treatment biopsies (Figure 1).

The MMP-2 levels showed a tendency to decrease in the antral mucosa, although the changes were relatively small, without meaningful differences between the treatment groups. MMP-2 levels were found to be unaffected in the corpus mucosa by successful eradication therapy (Table 1).

Gelatin zymography

In antral mucosa, active and latent MMP-9 levels decreased significantly after successful *H. pylori* eradication, compared with before treatment (Table 2). In corpus mucosa latent MMP-9 levels decreased significantly as well, whereas active MMP-9 levels showed a non-significant decrease. The three patients with persistent *H. pylori* infection also showed some decrease, though less impressive, in the active and latent MMP-9 levels after therapy. In contrast, the MMP-2 levels, active as well as latent, did not alter after therapy compared with those prior to therapy both in the *H. pylori* eradicated and in the persistent *H. pylori* positive group (data not shown), similar to the levels as determined by ELISA.

Table 2 - MMP-9 levels in gastric mucosa biopsy specimens of *H. pylori* positive patients before and after treatment as measured by zymography

Biopsy site	Therapy result	Latent MMP-9			Active MMP-9		
		Before	After	*P*-value	Before	After	*P*-value
Antrum	Successful, *n* = 34	116.5 ± 17.1	2.2 ± 1.5	< 0.001	25.7 ± 5.7	2.6 ± 2.3	< 0.001
	Unsuccessful, *n* = 3	102.7 ± 29.6	64.2 ± 46.5	NA	18.9 ± 5.1	7.8 ± 4.6	NA
Corpus	Successful, *n* = 34	25.0 ± 5.5	3.8 ± 1.7	<0.001	3.1 ± 0.9	1.3 ± 0.7	NS
	Unsuccessful, *n* = 3	30.3 ± 24.7	17.5 ± 9.5	NA	6.8 ± 6.8	0.7 ± 0.7	NA

Levels are expressed in AU / 5 µgr protein homogenate; NA : not applicable; NS : not significant

Bioactivity Assay (BIA)

Latent MMP-9 levels, as assessed by the BIA, also revealed that successful treatment resulted in a significant decrease in the gastric mucosa compared with those prior to treatment, whereas no major alterations were found in the patients in whom *H. pylori* was not eradicated after therapy (Table 3). With regard to the active MMP-9 levels similar results were obtained [antrum 5.6 ± 0.8 *vs*. 0.2 ± 0.1 (P<0.001) and corpus 2.1 ± 0.4 *vs*. 0.3 ± 0.1 (P<0.001), before and after successful treatment, respectively (n=53). The changes observed in the gastric mucosal MMP-9 levels, as determined by the BIA, of the successfully *H. pylori* eradicated patients again showed an identical pattern in the ranitidine and omeprazole treatment groups (data not shown). Latent MMP-2

Table 3 - Latent MMP-9 levels in gastric mucosa biopsy specimens of *H. pylori* positive patients before and after treatment as measured by BIA

Biopsy site	Therapy result	Latent MMP-9		
		Before	After	*P*-value
Antrum	Successful, *n* = 47/53	17.0 ± 1.8	1.6 ± 0.7	< 0.001
	Unsuccessful, *n* = 4	12.5 ± 4.5	7.3 ± 4.4	NA
Corpus	Successful, *n* = 49/53	5.9 ± 0.9	1.6 ± 0.6	< 0.001
	Unsuccessful, *n* = 3/4	3.7 ± 2.2	2.9 ± 1.5	NA

Levels are expressed in AU / mg protein; NA : not applicable

levels in the gastric mucosa were once more found to be hardly affected by the *H. pylori* treatment regimens (data not shown). Active MMP-2 was not assessed by the BIA based on the observations in the zymography, which revealed them to be very low or absent in the gastric mucosa homogenates.

Comparison of the three techniques used for MMP-9 measurement

Positive and significant correlations of the upregulated pre-treatment MMP-9 levels in gastric mucosa of *H. pylori* positive individuals were found between zymography, BIA and ELISA (Table 4). After successful eradication these correlations remained significant, although the MMP-9 levels were consistently decreased. Interestingly, before therapy all MMP-9 assessments revealed a significantly higher level in the antral mucosa compared with the corpus mucosa that completely disappeared after treatment, already noticeable in Table 1. However, the correlations between the overall MMP-9 levels measured by ELISA and the MMP-9 levels measured by the gelatin-zymography or the BIA after therapy are lower than before therapy, while correlations between gelatin-zymography and BIA remain high after therapy. This observation suggests alterations in the isoform composition of MMP-9 and/or in TIMP levels.

Table 4 - Correlation of MMP-9 levels in gastric mucosa of *H. pylori* positive patients before and after treatment as determined by ELISA, BIA and zymograpy

		MMP-9	
Assays	Biopsy site	Before	After
ELISA – BIA*	Antrum	0.89, <0.001[#]	0.27, 0.046
	Corpus	0.85, <0.001	0.44, <0.001
ELISA – zymography*	Antrum	0.81, <0.001	0.39, 0.018
	Corpus	0.65, <0.001	0.23, NS
BIA – zymography*	Antrum	0.74, <0.001	0.82, <0.001
	Corpus	0.85, <0.001	0.69, <0.001

* $32 \leq n \leq 57$, [#]Pearson correlation coefficient, *P*-value; NS : not significant

Discussion

H. pylori-associated chronic gastritis is recognized as a major risk factor for the development of gastric carcinoma [6, 7]. We previously showed alterations in the MMP-2 and/or MMP-9 levels in gastric tissues from patients with *H. pylori*-associated gastritis and from patients with gastric cancer [16, 17, 23]. In the present, uncontrolled, study we evaluated the effect of eradication therapy on these gastric MMP levels in patients with *H. pylori* gastritis. Latent, active and total MMP-9 levels decreased consistently and significantly after successful *H. pylori* eradication, in antrum as well as corpus mucosa, irrespective of the therapy regimen used. The

elevated levels remained unchanged, however, when treatment failed. The MMP-2 levels and activities in *H. pylori* positive patients did not change significantly by successful treatment.

MMP-9 in gastric mucosa is predominantly expressed by polymorphonuclear leukocytes, macrophages, (myo)fibroblasts, although *in vitro* studies also reported MMP-9 in epithelial cells [15, 24-27]. MMP-2 immunoreactivity was predominantly observed in stromal cells, inflammatory cells and epithelial cells [15, 24, 25, 28]. The MMP-9 levels in the antrum of our gastritis patients were found to be two- to four-fold higher compared with the corresponding corpus, dependent on whether it was a pan- or antral gastritis. This observation corresponds very well with our previously reported observation that the active inflammatory reaction, i.e., the number of infiltrated neutrophils, in the antrum is similarly more intense compared with the corpus mucosa [19]. The higher antrum inflammation is probably caused by a slow pyloro-cardial progression of gastritis as a consequence of a less dense *H. pylori* colonization of the corpus due to local acid production [29]. The presence and activation of these inflammatory cells are caused by mucosal cytokines, e.g. TNF-α and IL-8, which are increased in *H. pylori*-induced gastritis and are also capable of inducing the production of MMP-9 and less that of MMP-2 [30, 31]. This finding can be explained by the fact that the MMP-2 encoding gene lacks an AP-1 binding site that prevents activation by TNF-α or IL-β. MMP-9, however, is an inducible matrix metalloproteinase, in contrast to MMP-2 that is expressed more constitutively [9].

With successful *H. pylori* eradication, the antigen responsible for the immune reaction is removed, leading to a slow but progressive decrease in both the active and chronic component of the gastric mucosal inflammation, including reduction of cytokine production [32-34]. In our population of patients, both forms of inflammation also decreased significantly in both antrum and corpus after successful treatment of the *H. pylori* infection [18, 19]. This decrease in inflammation was accompanied with a considerable and significant decrease of latent, active and total MMP-9, particularly in the antrum. Our results are in line with a preliminary immunohistochemical study that showed a significant decrease of enhanced MMP-9 expression in epithelial cells and fibroblasts - but not in macrophages - after *H. pylori* eradication and no alterations in MMP-9 expression where eradication failed [35]. Another immunohistochemical study, however, reported an increase in MMP-9 staining of surface mucous cells and pyloric glands of gastric antral biopsies from patients after *H. pylori* eradication [36]. The observations that the MMP-9 levels in the gastric mucosa of the unsuccessfully treated patients remain elevated suggest a direct relationship between *H. pylori* presence and MMP-9 level. Yet, in some of our assessments, e.g. zymography and BIA, some decrease in MMP-activity was noticeable in the *H. pylori* persistent patients. Probably, the acid-reducing drugs used might have an intrinsic inhibitory effect on the MMPs,

as previously shown by the inhibitory effect of H_2-receptor antagonists on matrix metalloproteinases in rat gastric tissues with acetic acid-induced gastric ulcers [37, 38]. On the other hand, alterations in the level or activity of TIMPs, the endogenous MMP inhibitors, cannot be excluded but were not assessed in the present study.

Improvement and normalization of the chronic inflammatory reaction in the stomach after successful *H. pylori* eradication is accompanied by a reversal of many altered mucosal parameters that have been associated with gastric cancer and its prognosis, e.g. growth factors and cytokines [31-33], plasminogen activators [18, 39] and superoxide dismutases [19, 40]. Patients with *H. pylori*-associated chronic gastritis are predisposed for gastric carcinoma but its remains unclear whether eradication therapy also results in a reduction of gastric cancer incidence, since most of the *H. pylori* positive patients do not develop cancer, and inflammation and cancer diversity genes might play a more important role [41, 42]. Apparently also higher tissue levels of MMP-2, as in the tumors [17, 24], are required in combination with elevated MMP-9 levels for the development of *H. pylori* gastritis to carcinoma. Our study is not conclusive in that respect due to the absence of major alterations in the MMP-2 levels. Larger studies, including pathogenicity classification of the *H. pylori* strains, are needed to get a better insight into the relevance of changes in the MMP expression in the development of gastric cancer. In addition, genetic susceptibility might also play a role, as illustrated by the MMP-$7_{-181A>G}$ gene polymorphisms which has recently been found to be associated with both gastric ulcerogenesis in *H. pylori* infection and gastric cancer, which provides a potential genetic link and implicates other MMPs in the association between both disorders [43, 44].

In conclusion, the *H. pylori*-associated increased MMP-9 levels in antrum and corpus mucosa decrease significantly by successful eradication of *H. pylori*. No major changes occurred in the MMP-2 levels and activities by eradication therapy and in the MMP-9 levels when eradication failed.

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CHAPTER 4

Tissue levels of matrix metalloproteinases MMP-2 and MMP-9 are related to the overall survival of patients with gastric carcinoma

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Summary

Proteinases are involved in tumour invasion and metastasis. Several matrix metalloproteinases (MMPs) have been shown to be increased in various human carcinomas. We assessed the levels of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in 50 gastric carcinomas and corresponding mucosa using quantitative gelatin zymography. Both MMP levels were significantly enhanced in gastric carcinomas compared with adjacent mucosal tissue, showed a relatively poor intercorrelation and no relation was found with histopathological carcinoma classifications according to Laurén, WHO and tumour-node-metastasis (TNM). Cox's multivariate proportional hazards analyses revealed that high carcinomatous MMP values are of prognostic significance for a poor overall survival of the patients, independent of the major clinicopathological parameters.

Introduction

The process of carcinogenesis involves sequential breakdown of extracellular matrix by a variety of proteolytic enzymes [1]. Gelatinases, collagenases and stromelysins are metalloproteinases (MMP-2), which are able to solubilise collagens in basement membranes and extracellular stroma [2]. This local proteolysis enables tumour cells to penetrate normal surrounding tissue. Immunohistochemical and *in situ* hybridisation studies in human gastrointestinal neoplasias have shown that these carcinomas contain enhanced amounts of matrix metalloproteinases [3-5]. The enhanced proteolytic capacity of tumour tissues is confirmed by studying tissue homogenates, using quantitative methods like activity assays, and ELISAs [6-8]. Some *in vitro* and *in vivo* experiments showed that matrix metalloproteinase levels were related to the invading and metastatic potential of colorectal cancer [7, 9]. Moreover, plasma levels of some MMP-2 were found to be enhanced in patients with colonic cancer [10].

In this study we used a relatively straightforward method, gelatin zymography, to evaluate the presence of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in stomach carcinomas and adjacent mucosa from 50 patients, from whom clinical and histopathological data concerning patients and carcinomas were available. Quantitative zymography has been shown previously to be an extremely reliable and sensitive technique for the detection of gelatinases [11, 12]. Moreover, this method of detection distinguishes proteinases in the proenzyme and the active form. The amounts of MMP-2 were related to several types of gastric tumour staging systems, including the classifications of Laurén, WHO and TNM. The prognostic significance of the MMP-2 and MMP-9 levels for the survival of patients with a gastric carcinoma was evaluated using Cox's proportional hazards method in univariate analysis, and also multivariately by addition to a broad selection of established clinicopathological variables.

Patients, materials and methods

Patients

Fresh tissue was obtained from 50 patients who underwent resection with curative intent for primary gastric cancer at the Department of Oncology Surgery, University Hospital Leiden, as previously described [13]. Representative samples of the carcinoma and macroscopically normal mucosa, taken 5 – 10 cm from the tumour, were frozen and stored at –70°C until extraction. Pathological and histological data of the tissues were re-evaluated by one pathologist (JvK). The patients entered the study at the date of surgery, did no receive adjuvant (chemo) therapy, and were clinically checked twice a year. Follow-up had to be at least 2 years and ended in the event of death or when

still alive the last follow-up date before the common closing date (follow-up range 0.5 – 81 months).

Tissue extraction and protein concentration

Tissue specimens were homogenised in 0.1 M Tris-HCl, 0.1% (v/v) Tween 80 as described extensively previously [13-15]. Protein concentrations of the supernatants were determined by the method of Lowry *et al*. [16].

Gelatin-zymography

Presence of active and latent forms of matrix metalloproteinases was analysed by zymography on 10% polyacrylamide gels containing 2% gelatin and overnight incubation at 37°C, as described previously [17]. Sample volumes were adjusted to obtain a uniform protein content of 20 µg per sample. The gels were stained with Coomassie brilliant blue R-250, dried between sheets of cellophane, and the degree of gelatin digestion was quantified using an LKB Ultroscan XL enhanced laser densitometer (633 nm). Two amounts (12 and 24 µg protein, S_1 and S_2 respectively) of an internal standard preparation, i.e. a homogenate of a colonic carcinoma containing both MMP-2 and MMP-9, were included on each gel for correction of intergel variation and as reference for the expression in arbitrary units (AU). This zymographic analysis was highly linear over an at least 20-fold range (i.e. 2 – 40 µg protein per sample and was validated for MMP-9 by an established ELISA [18] in 30 diverse gastrointestinal tissue homogenates yielding a good correlation between these assays ($0.65 < r < 0.77$, $P < 0.0001$).

Statistical analyses

Group means are given as mean ± s.e.m. Differences between groups were tested for significance using paired Student's *t*-test with separate variance estimate if the standard deviations were significantly different according to the *f*-test. Optimal cut off analysis was performed by stepwise univariate Cox's proportional hazards analyses. Univariate and multivariate survival analyses were performed using Cox's proportional hazards method (EGRET statistical package, SERC Corp., Seattle, WA, USA) [19]. Overall survival curves were constructed according to the method of Kaplan and Meier [20]. Differences were considered significant when $P < 0.05$.

Results

The characteristics of the 50 gastric cancer patients revealed that most of the patients were males (38 patients, i.e. 76%) and had died during follow-up (76%, 38/50), although the deceased patients were not significantly older [67.2 ± 1.8 years ($n = 38$)

Table I - Univariate Cox's proportional hazards proportional hazards analysis of clinicopathological parameters in relation to overall survival of patients with gastric cancer

Parameter	Number of patients	Medium survival time (months)	Survival (%)	Hazard ratio (*P*-value)
Gender				
male *vs* female	38-12	16.0-13.0	26.3-16.7	1.1 (NS)
Age (years)				
<66.3 *vs* ≥66.3 (median)	25-25	18.4-10.1	20.0-28.0	1.2 (NS)
Laurén classification				
Diffuse/mixed *vs* intestinal	18-31	27.0-11.3	33.3-16.1	1.6 (NS)
WHO differentiation				
Well/moderately *vs* poorly	34-15	15.0-27.1	14.7-40.0	0.6 (NS)
TNM				
Stage I+II *vs* stage III+IV	34-16	18.3-15.0	29.4-12.5	1.3 (NS)
Localisation				
Antrum *vs* other	23-27	18.3-12.3	30.4-18.5	1.6 (NS)
Diameter				
≤5 cm *vs* >5 cm	28-22	18.0-12.5	25.0-22.7	1.1 (NS)
Eosinophils				
Many *vs* moderate/few	7-43	4.3-16.4	0.0-27.9	0.4 (0.02)
Intestinal metaplasia in mucosa Absent *vs* present	18-32	11.5-18.0	11.1-31.3	0.5 (NS)

NS, not significant

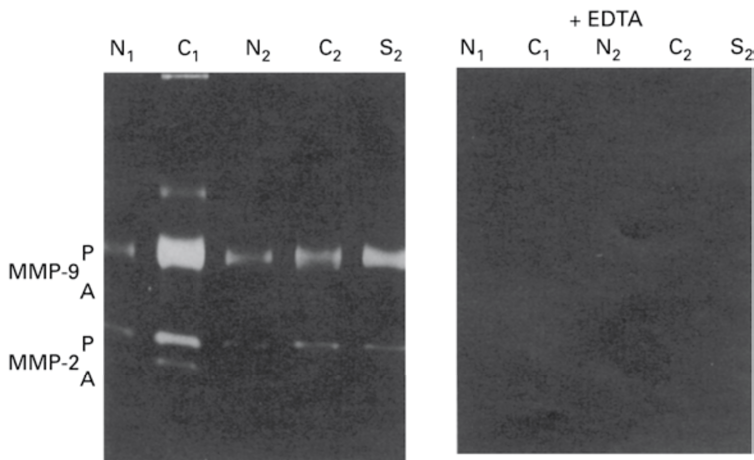

Figure 1. Example of the gelatin zymograms used for the MMP-2 and MMP-9 quantitation by laser densitometry, as described in Materials and methods. Complete inhibition of the MMP activities was achieved by overnight incubation in the presence of 50 mM EDTA. Numbers indicate pairs of tissue from one patient. N, gastric mucosa; C, gastric carcinoma; S, standard (reference). MMPs: P, pro-enzyme; A, active enzyme.

Table II Levels of matrix metalloproteinases MMP-2 and MMP-9 in mucosa and carcinomas of 50 patients with gastric cancer

	Mucosa	Carcinoma	*P*-value Paired *t*-test
MMP-2			
Total	1.50 ± 0.11	2.63 ± 0.23	<0.001
Pro-form	1.24 ± 0.11	1.90 ± 0.16	<0.001
Active	0.26 ± 0.03	0.73 ± 0.10	<0.001
MMP- 9			
Total	3.72 ± 0.23	5.92 ± 0.32	<0.001
Pro-form	3.18 ± 0.21	4.99 ± 0.25	<0.001
Active	0.54 ± 0.08	0.93 ± 0.09	0.001

Mean ± s.e. The MMPs were quantified using gelatin-zymography and subsequent laser densitometry. Values are expressed in arbitary units

vs 66.0 ± 4.5 years (*n* = 12)]. All the clinicopathological parameters assessed were dichotomised as illustrated in Table I. Subdivision according to established histological tumour classification systems was found to have no major prognostic relevance in this group of patients, although overall survival decreased with increasing TNM stage [i.e. I, 43% (6/14); 20% (4/20); III, 17% (2/12); IV, 0% (0/4)]. Including all the other clinicopathological parameters evaluated, only the presence of many eosinophilic cells in the carcinomas was significantly associated with a worse survival, exemplified by a shorter median survival time and a low percentage survival of the patients (Table I).

The mean levels of matrix metalloproteinases MMP-2 and MMP-9, as determined by EDTA-inhibitable gelatin-zymography (Figure 1), were significantly higher in carcinomas than in histologically confirmed tumour-free adjacent mucosa of the stomach, irrespective of MMP type or activity state (Table II). Of the carcinomas, 82% (41/50) contained more total MMP-2 and 80% (40/50) contained more total MMP-9 than their corresponding mucosa, i.e. ratios higher than 1, as illustrated in Figure 2. The enhanced amounts of MMPs in the carcinomas were not significantly correlated to any of the histological gastric tumour classification systems, although the carcinomas that were superficially invasive showed the lowest total MMP levels (MMP-2, 1.28±0.34; MMP-9, 2.49 ± 1.18; in AU, *n* = 4), and were similar to the mucosal levels. The total levels of MMP-2 and MMP-9 showed a relatively poor intercorrelation (mucosa r = 0.19, NS; carcinomas r = 0.34, *P* = 0.01). For each of the MMP parameters in mucosa and carcinoma tissues the optimum cut-off values were determined using Cox's proportional hazards analyses (Table III). In mucosa a significant cut-off value was found only for the active form of MMP-9 and indicated that a high level was associated with a good prognosis. In contrast, for the carcinomas, the total and the pro-forms of MMP-2 and MMP-9, as well as the active form of MMP-2 showed significant cut-off values revealing that

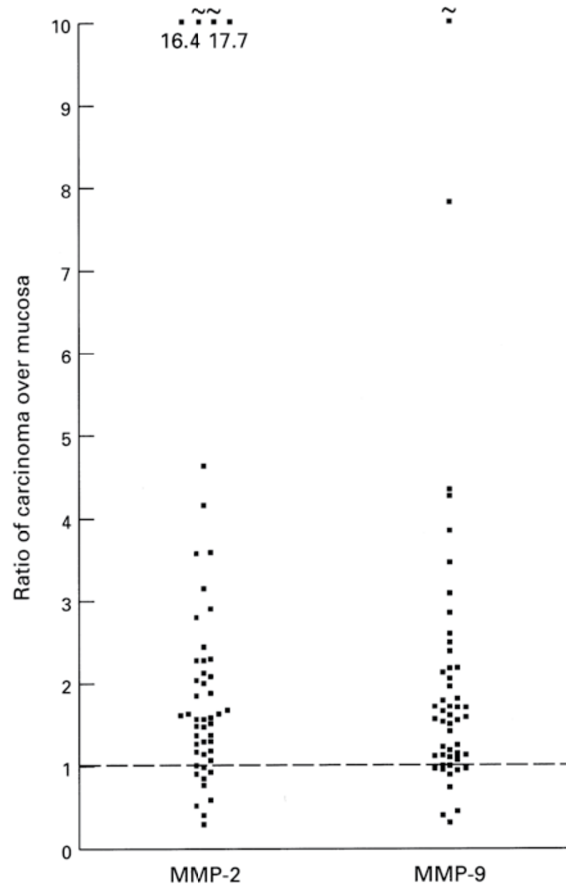

Figure 2. Individual data of the total MMP-2 and total MMP-9 ratio, carcinoma over mucosa, of the 50 gastric cancer patients. Dotted line indicates a ratio of 1, i.e. MMP level in carcinoma is identical to that of the gastric mucosa.

high levels indicated poor prognosis. Representative Kaplan-Meier curves for overall survival according to the cut-off points for total MMP-2 and MMP-9 are shown in Figures 3 and 4. Table III shows the hazard ratios of all the significant MMP parameters according to Cox's proportional hazards analyses. For the multivariate analyses the MMP parameters were separately evaluated by adjusting to all clinicopathological variables as listed in Table I. All the MMP parameters kept their prognostic significance in the multivariate analyses.

Table III - Uni- and multivariate Cox's proportional hazards analyses of MMP-2 and MMP-9 in gastric mucosa and gastric carcinomas related to overall survival of the patients

Parameter[a]	Number of patients	Median survival time (months)	Survival (%)	Hazard ratio (*P*) Univariate	Hazard ratio (*P*) Multivariate
Mucosa					
MMP-9 active <0.36 *vs* >0.36	25-25	8.4-27.4	16.0-32.0	0.4 (0.02)	0.3 (0.02)
Carcinoma					
MMP-2 total <4.00 *vs* >4.00	42-8	18.2-10.0	28.6-0.0	2.6 (0.02)	2.5 (0.05)
MMP-2 pro-form <2.82 vs >2.82	42-8	18.2-10.0	28.6-0.0	2.6 (0.02)	2.9 (0.03)
MMP-2 active <0.55 *vs* >0.55	27-23	27.4-10.4	37.0-8.7	2.1 (0.03)	3.1 (0.02)
MMP-9 total <7.25 *vs* > 7.25	35-15	18.4-10.1	31.4-6.7	2.0 (0.04)	2.1 (0.05)
MMP-9 pro-form <5.75 *vs* >5.75	33-17	27.1-9.3	33.3-5.9	2.6 (0.006)	2.8 (0.01)

Multivariate analyses were performed by adjusting the separate MMP parameters to all clinicopathological parameters indicated in Table II. [a]In arbitary units.

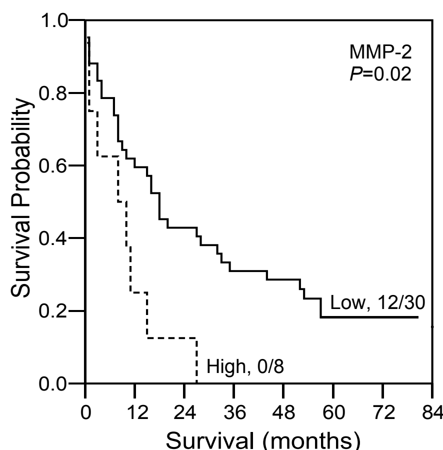

Figure 3. Kaplan-Meier overall survival curve for total MMP-2 levels in gastric carcinomas. MMP-2 values were evaluated using gelatin zymography and subsequent laser densitometry and are expressed in arbitrary units. High and low levels of MMP-2, cut-off point 4.0, were determined by Cox's univariate proportional hazards analysis. Values indicate the number of patients alive/deceased at the end of follow-up.

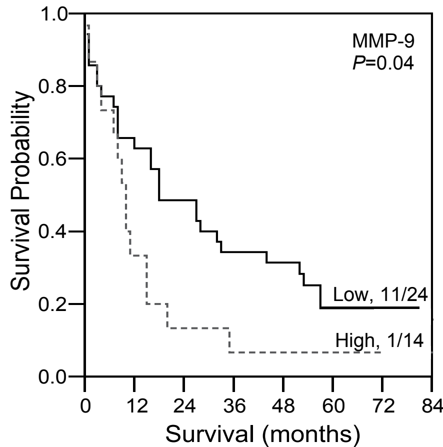

Figure 4. Kaplan-Meier overall survival curve for total MMP-9 levels in gastric carcinomas. MMP-9 values were evaluated using gelatin zymography and subsequent laser densitometry and are expressed in arbitrary units. High and low levels of MMP-9, cut-off point 7.25, were determined by Cox's univariate proportional hazards analysis. Values indicate the number of patients alive/deceased at the end of follow-up.

Discussion

Several proteolytic enzymes are involved in carcinogenesis. Various studies have shown, for instance, high concentrations of plasminogen activators, cathepsins and matrix metalloproteinases in different types of human carcinomas [1-10]. In the present study we show that in a majority of gastric carcinomas the MMP-2 and MMP-9 levels are significantly higher in the corresponding gastric mucosa, irrespective of the activity state of the enzymes. Moreover, our observation that the more deeply invasive carcinoma contain high levels of MMP's, whereas the superficially invasive tumours do not show more MMP than the corresponding mucosa, is in agreement with recent immunohistological data in which MMP-2 was found to be higher in advanced *vs* early gastric tumours [5]. The levels of MMP-2 and MMP-9 showed a relatively poor intercorrelation, both in gastric mucosa and in carcinomas, suggesting an independent expression pattern for both proteinases, which is probably related to differences in the cellular origin of these enzymes [2], but this was not assessed in the present study. Recently, the evaluation in carcinomatous tissue of some components of the plasminogen activation cascade, another important proteolytic system in carcinogenesis, has been found to be of significant value for the prognosis of cancer patients [15, 21-26]. Although the number of patients in the present study is relatively low, the results clearly show that high levels of MMP-2 and MMP-9 in stomach carcinomas are associated with a poor overall survival, which has never been reported before. The

distinction between total, active and pro-form of MMPs in our study, as one of the important advantages of the zymographic analysis, seems to be particularly useful for MMP-2. The interpretation of the prognostic significance of MMP-9 in mucosa from patients with a gastric carcinoma is difficult. However, high levels of tissue-type plasminogen activator activity in normal colorectal and gastric mucosa were also found to be associated with a good prognosis in colorectal and gastric cancer patients [13, 24].

The results of this study could have important clinical implications. Firstly, the prognostic significance of both MMPs in carcinomatous tissue is striking, especially in comparison with the relatively disappointing performance of established parameters like TNM and Laurén classification or diameter of the carcinoma. Therefore these proteolytic parameters may be suitable as prognosticators for the selection of patients for adjuvant therapy. Secondly, this study might give some rationale for therapeutic intervention with matrix metalloproteinase inhibitors, which has recently been demonstrated to be effective in patient-like orthotopic human tumour models in nude mice [27, 28].

Acknowledgements

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CHAPTER 5

Matrix metalloproteinase-2 is a consistent prognostic factor in gastric cancer

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Abstract

In a pioneer study, we showed 10 years ago that enhanced tissue levels of the matrix metalloproteinases (MMPs) MMP-2 and MMP-9 in gastric cancers, as determined by zymography, were related with worse overall survival of the patients. To corroborate these observations, we now assessed MMP-2 and MMP-9 with new techniques in an expanded group of gastric cancer patients ($n = 81$) and included for comparison MMP-7, MMP-8 and the tissue inhibitors of MMPs, TIMP-1 and -2. All MMPs and TIMP-1 were significantly increased in tumour tissue compared to normal gastric mucosa. Matrix metalloproteinase-7, -8 and -9, and the TIMPs showed some correlations with the clinicopathologic parameters TNM, WHO and Laurén classification, but their levels were not related with survival. Regardless of the determination method used, that is, enzyme-linked immunosorbent assay or bioactivity assay, an enhanced tumour MMP-2 level did not show a significant correlation with any of the clinicopathological parameters, but was confirmed to be an independent prognostic factor in gastric cancer.

Introduction

A decade ago, we were the first to report that the levels of matrix metalloproteinase (MMP)-2 and MMP-9 in human gastric carcinoma tissues were enhanced and related to the survival of the patients, using a simple but laborious zymography technique in a relatively small group of patients (Sier *et al*, 1996). Matrix metalloproteinases are believed to play an important role in carcinogenesis via the degradation and remodelling of tumour surrounding extracellular matrix, which could explain the association with survival (Zucker *et al*, 2000; McCawley and Matrisian, 2001; Polette *et al*, 2004). We concluded that measuring MMPs could have clinical value as indicators for gastric carcinoma patients who needed adjuvant therapy and that inhibitors of MMPs might be useful for therapeutic intervention. Several accomplishments have been made since. The prognostic value of MMPs for gastric carcinoma patients has been confirmed in several other studies (Allgayer *et al*, 1998; Zhang *et al*, 2003), and clinical trials testing the effect of MMP inhibitors for patients with various types of cancer were performed, with variable success (Zucker *et al*, 2000; Bramhall *et al*, 2002).

In general, MMPs are secreted as inactive pro-enzymes, activated by proteolytic cleavage, and controlled in their activity by interaction with inhibitors. Disturbances in these processes are of eminent importance in tumour invasion and metastasis (McCawley and Matrisian, 2001; Polette *et al*, 2004). In the present more comprehensive study, we extended our MMP analyses in the same group of patients and compared the results with those obtained with a new and more recent group of patients. Furthermore, instead of zymography, which identifies isoforms, we now used recently established quantitative bioactivity assays (BIAs) and specific antigen enzyme-linked immunosorbent assays (ELISAs) for MMP-2 and MMP-9. Moreover, we compared the prognostic value of MMP-2 and MMP-9 with those of MMP-7 and MMP-8 and expanded the study by determination of the inhibitors TIMP-1 and TIMP-2. In addition, because of the increasing age of the patients and the length of the follow-up, we now used tumour-associated survival.

Patients, materials and methods

Patients and study design

Fresh tissue specimens of 81 patients (21 female and 60 male subjects, mean age 65.9 years, range 35.10 –91.33), who underwent resection for primary gastric adenocarcinoma at the Department of Oncologic Surgery of the Leiden University Medical Centre, were collected prospectively. Immediately after resection, fresh samples from the mid-central, non-necrotic part of the carcinoma and/or from distant normal mucosa,

taken approximately 10 cm from the tumour, were snap frozen and stored at 70° C until extraction, to be used for research purposes. Various clinicopathological data were evaluated or collected from patient files. All carcinomas were classified according to the TNM classification (Hermanek and Sobin, 1992) and localisation and also diameter of the tumour, differentiation grade, WHO, Borrman, and Laurén classification, as well as the presence of intestinal metaplasia in the normal gastric mucosa, as revised by a gastroenterologist (FK) and a pathologist (JvK). All patients entered the study at operation date, and the patient's time experience ended in the event of death or, when still alive, at the common closing date. The minimal follow-up was 33 months with a decreasing overall survival according to TNM stage, that is, from TNM I (52.2%, n = 23), to TNM II (26.9%, n = 26), to TNM III (28%, n = 25), and to TNM IV (0%, n = 7).

Tissue preparation and protein concentration

Homogenisation of tissue specimens and determination of protein concentrations were performed as described previously (Sier *et al*, 1996).

Metalloproteinase-2 and -9 activity assays

Quantitative gelatin zymography and BIAs for MMP-2 and MMP-9 were carried out as described before (Sier *et al*, 1996; Hanemaaijer *et al*, 2000). Active and activatable (pro) MMP-2 and MMP-9 were determined with the BIA in 96-well plates, coated with monospecific antibodies to the MMPs, sample/standard incubation overnight and detection by modified MMP-sensitive pro-urokinase in combination with peptide substrate S-2444 and measurement of absorbance change at 405 nm over time. Activation of pro-MMPs was achieved by incubation with p-aminophenyl-mercuric acetate.

Enzyme-linked immunosorbent assay for MMP-2, MMP-7, MMP-8, MMP-9, TIMP-1 and TIMP-2

Antigen levels of MMP-2 and MMP-9 were determined using previously described ELISAs (Hanemaaijer *et al*, 1998). In brief, the same catching antibodies were used as for the BIAs. Next, appropriate dilutions of tissue homogenates were incubated overnight at 4°C. Immunodetection of MMP-2 and MMP-9 was performed directly or indirectly with in-house anti-MMP-2 and -MMP-9 biotinylated-polyclonal antibodies. Avidin – horseradish peroxidase and 3,3′,5,5′ tetramethyl benzidine were used for the colouration reaction. The respective amounts of MMP-2 and MMP-9 were calculated from standard curves. The concentrations of MMP-7, TIMP-1 and TIMP-2 antigens were determined using commercial ELISAs according to the manufacturer's instructions (R&D Systems Europe, Abingdon, UK). The amount of MMP-8 was measured using a previously described ELISA (Bergmann *et al*, 1989).

Statistical analysis

Differences between normal and tumour values for all parameters were calculated using the Wilcoxon signed rank test. For the survival analyses, the clinicopathological parameters were dichotomised as described previously unless indicated elsewhere. Cutoff points for MMP data were optimised or medians were used. Univariate and multivariate survival analyses were performed with the Cox proportional hazards model, using the SPSS Windows Release 12.0.1. statistical package (2004, SPSS Inc., Chicago, IL, USA). Multivariate survival analyses were performed using the Cox proportional hazards method by separately adding the significant MMP variables to the dichotomised clinicopathological parameters. Overall and tumour-related survival curves were constructed using the method of Kaplan and Meier including the Log-rank test. Differences were considered significant when $P \leq 0.05$.

Results

Although quantitative zymography is a reliable and sensitive technique to identify active and latent isoforms of MMP-2 and MMP-9, it is a laborious assay to perform. Therefore, we compared the previously obtained zymography data for MMP-2 and MMP-9 with the results from more practicable and sophisticated immunoassays, that is, BIAs and ELISAs. Table 1 shows an overview of the correlation coefficients and *P*-values for the different assays (samples $n = 100$). The total zymography data, which consist of the sum of active and pro-form bands, correlated significantly with the total BIA and ELISA levels for both MMP-2 ($0.312 < \rho < 0.533$, $P \leq 0.003$) and even better for MMP-9 ($0.558 < \rho < 0.817$, $P < 0.001$). The latent pro-forms of MMP-2 and MMP-9, separately detected by zymography and BIA, also correlated significantly. No correlation between both assays was found, however, for active MMP-2 or MMP-9, indicating that the active isoform as identified by the very sensitive zymography is not necessarily functionally active in the less-sensitive BIA, probably through interaction with inhibitors.

The levels of MMP-2 and MMP-9 as detected with the BIAs and ELISAs in normal mucosa and tumour tissue in the expanded group of 81 gastric carcinoma patients are shown in Table 2. Carcinomas contained significantly higher MMP-2 and MMP-9 levels in antigen as well as activity than adjacent normal tissue. Particularly remarkable is the presence of more active MMP-2, but not of active MMP-9, in the tumour tissue homogenates. The most impressive enhancement (>20-fold) in carcinomas compared to normal tissue, however, was noted for MMP-7 (Table 2). Matrix metalloproteinase-8 and TIMP-1 were also significantly increased, whereas tumour TIMP-2 levels were found not to be enhanced. Interestingly, a striking difference was observed in the correlation

Table 1 - Spearman's ρ for three different assays used for the detection of MMP-2 and MMP-9 in 50 normal/tumour pairs of tissue homogenates from gastric cancer patients

	BIA-total	BIA-pro	BIA-active	ELISA
MMP-2				
Zymo				
Total	0.312 0.003	0.283 0.008	0.203 NS	0.533 <0.001
Pro	0.356 <0.001	0.325 0.002	0.233 <0.030	0.439 0.003
Active	-0.010 NS	-0.021 NS	0.053 NS	0.481 <0.001
BIA				
Total	1	0.982 <0.001	0.340 0.001	0.505 <0.001
MMP-9				
Zymo				
Total	0.558 <0.001	0.604 <0.001	0.100 NS	0.770 <0.001
Pro	0.533 <0.001	0.569 <0.001	0.089 NS	0.740 <0.001
Active	0.417 <0.001	0.462 <0.001	0.069 NS	0.523 <0.001
BIA				
Total	1	0.930 <0.001	0.538 <0.001	0.817 <0.001

Zymo = zymography, BIA = bioactivity assay, ELISA = antigen; MMP = matrix metalloproteinase. The white values/boxes are the correlations between similar entities, for example, total-total, pro-pro, active-active with different techniques or total with ELISA.

between the primary MMP – TIMP interactor antigen levels, that is, MMP-9 with TIMP-1 (ρ=0.358, P<0.0005) and MMP-2 with TIMP-2 (ρ=0.085, NS). The levels of MMPs and TIMPs were also evaluated for correlation with all the clinicopathological parameters. Tumour levels of MMP-2, TIMP-1 and TIMP-2 did not show significant correlations with any of these parameters. The mean MMP-7 levels increased stepwise with TNM classification (Figure 1) and were significantly enhanced in Laurén's intestinal-type carcinomas compared to diffuse or mixed types (56±16 *vs* 34±22, P<0.02). Matrix metalloproteinase-8 levels were enhanced in Laurén's intestinal-type tumours (402±72 *vs* 178±29 ng mg^{-1} protein, P<0.006) and differentiated tumours (393±67 *vs* 163±29ng mg^{-1} protein, P<0.002) according to the WHO classification. Matrix metalloproteinase-9 levels showed a similar enhancement for Laurén's intestinal-type carcinomas (BIA total activity 140 *vs* 99 U mg^{-1} protein, P<0.02; ELISA 29 *vs* 17 ng mg^{-1} protein, P<0.01) and differentiated tumours (BIA total activity 133±13 *vs* 104±16 U mg^{-1} protein, NS;

Table 2 - Antigen levels (ng mg-1 protein) of MMP-2, MMP-7, MMP-8 and MMP-9 and of inhibitors TIMP-1 and TIMP-2 in normal mucosa and carcinoma of 81 patients with gastric cancer

	Mucosa	Carcinoma	*P*-value
MMP-2			
Antigen	4.7 ± 0.4	17.0 ± 2.0	≤0.001
Total activity[a]	81.1 ± 23.6	185.7 ± 45.5	≤0.001
Pro-form[a]	78.9 ± 23.6	181.1 ± 45.3	0.001
Active[a]	2.3 ± 0.5	4.7 ± 1.1	0.02
MMP-9			
Antigen	9.0 ± 0.9	24.7 ± 2.3	≤0.001
Total activity[a]	67.5 ± 6.0	128.8 ± 11.3	≤0.001
Pro-form[a]	59.9 ± 5.6	117.1 ± 0.1	≤0.001
Active[a]	7.6 ± 1.5	9.5 ± 2.1	NS
MMP-7	2.0 ± 0.5	47.1 ± 12.4	0.002
MMP-8	95 ± 12	319 ± 47	≤0.001
TIMP-1	8.0 ± 0.8	16.9 ± 1.3	≤0.001
TIMP-2	5.9 ± 0.2	6.3 ± 0.4	NS

Mean ± s.e.m. Bioactivity assay levels of MMP-2 and MMP-9 are expressed as units per mg protein. [a]As determined by BIA. MMP = matrix metalloproteinase; BIA = bioactivity assay; TIMP = tissue inhibitors of MMP.

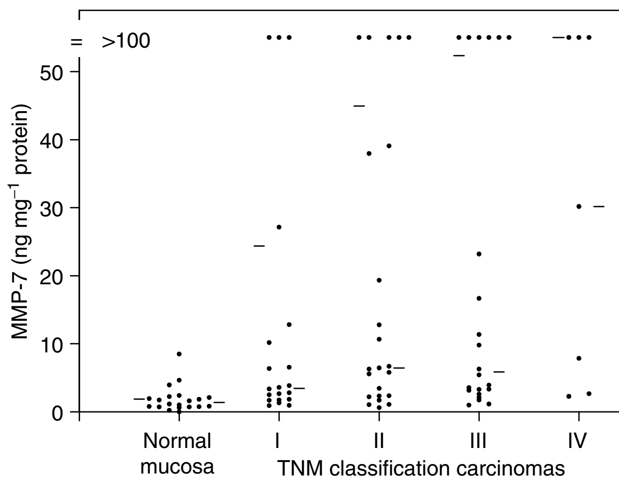

Figure 1. Relation between MMP-7 antigen levels and TNM classification in gastric carcinomas. The mean and median for the subgroups are indicated by bars on, respectively, the left- and right-hand side of each column.

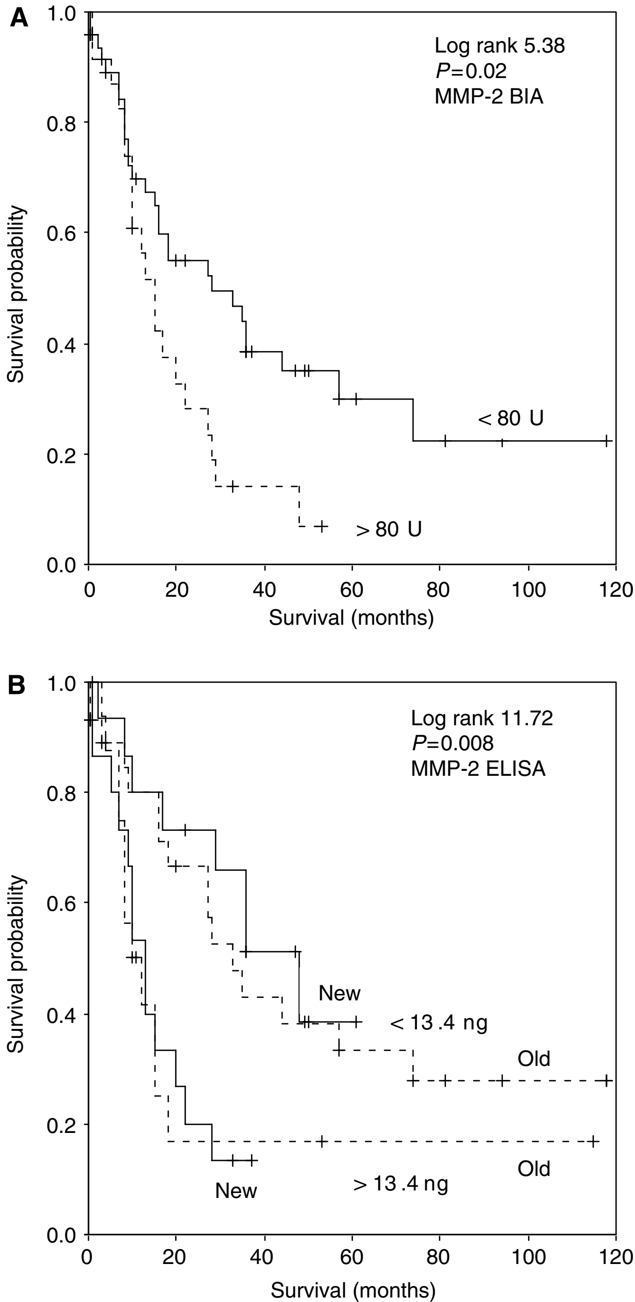

Figure 2. Kaplan–Meier tumour-related overall survival curves for (A) MMP-2 BIA total activity, (B) MMP-2 ELISA old *vs* new gastric cancer patient groups, with the cutoff levels from the Cox analyses.

Table 3 - Uni-and multivariate Cox's proportional hazards analyses of MMP-2, determined by ELISA and BIA, and clinicopathological parameters in relation to the overall tumour-related survival of 81 patients with gastric cancer

Univariate					Multivariate		
Parameter	*n*	HR	CI 95%	*P*	HR	CI 95%	*P*
Gender							
Male *vs* female	60/21	1.384	0.768 –2.494	NS	1.767	0.935– 3.342	NS
Age							
Median (66 years)	40/41	1.313	0.764 –2.255	NS	1.467	0.775– 2.774	NS
TNM							
I	23/81	1	—		1	—	
II	26/81	3.133	1.360 –7.222	0.007	4.001	1.510– 10.60	0.005
III	25/81	3.021	1.305 –6.991	0.010	3.557	1.290– 9.813	0.014
IV	7/81	7.387	2.495 –21.86	0.000	20.416	4.992– 83.49	0.000
Laurén							
Diffuse/mixed *vs* intestinal	30/50	0.889	0.516 –1.531	NS	1.152	0.353– 3.756	NS
WHO differentiation							
Well *vs* poor	54/26	1.133	0.650 –1.975	NS	1.270	0.370– 4.363	NS
Borrmann							
I+II vs III+IV	55/24	1.118	0.609 –2.053	NS	0.761	0.386– 1.502	NS
Localisation							
Cardia *vs* rest	36/45	0.573	0.330 –0.993	0.034	0.330	0.159– 0.682	0.003
Diameter tumour							
<5 *vs* >5 cm	47/34	1.048	0.608 –1.808	NS	0.622	0.337– 1.149	NS
Eosinophils							
Few *vs* many	56/24	1.035	0.568 –1.886	NS	1.743	0.806– 3.766	NS
Intestinal metaplasia							
Absent *vs* present	39/42	0.490	0.280 –0.858	0.013	0.706	0.379– 1.315	NS
Carcinoma							
MMP-2 ELISA							
<13.4 *vs* >13.4 ng mg^{-1} protein	45/31	2.611	1.455 –4.686	0.001	2.620	1.249– 5.494	0.011
MMP-2 BIA							
<80 *vs* >80 U mg^{-1} protein	49/23	1.974	1.089 –3.577	0.025	1.493	0.655– 3.404	NS

HR = hazard ratio; CI = confidence interval.

ELISA 28±3 *vs* 17±3 ng mg^{-1} protein, $P<0.02$). Matrix metalloproteinase-9 total activity showed a stepwise decrease with TNM classification, which did not reach significance (I, 145±27; II 127±18; III, 120±20; IV, 114±28 U mg^{-1} protein).

For tumour-associated survival analyses, all MMP and TIMP parameters in tumour homogenates were evaluated for optimal cutoff points using the log rank test. Significant cutoffs were only found for the MMP-2 levels by BIA and ELISA (Figures 2A and B).

No significant association for MMP-7, MMP-8, MMP-9, TIMP-1 and TIMP-2 with tumour-associated survival was found according to stepwise univariate Cox analyses and thus the medians were used (hazard ratio and 95% confidence interval ranges of the median levels varied from 0.801 to 1.257 and from 0.445 to 2.307, respectively). High MMP-2 levels determined by BIA as well as ELISA were significantly associated with worse survival, but in multivariate analyses with the clinicopathological parameters, only the MMP-2 ELISA kept its independent prognostic value (Table 3). The consistent prognostic relevance of MMP-2 is underlined by Figure 2B, in which the old group of patients (n = 50) and the more recent patients group (n = 31) are independently subdivided based on a low or high MMP-2 antigen content of the carcinoma, using the same cutoff value. Similar results were obtained with the BIA data (not shown).

Discussion

The present study corroborates our previous finding of increased MMP-2 in gastric cancer. The high MMP-2 antigen and activity levels were significantly associated with worse survival according to univariate Cox proportional hazards analysis. In the multivariate analysis, including a broad selection of clinical parameters, the MMP-2 antigen level kept its independent prognostic value, but the significance for the MMP-2 BIA activity level of the carcinomas was lost. The optimal cutoff point for MMP-2 antigen calculated for survival prognosis in the old group of patients was similarly predictive in the new group of patients, indicating the strength of MMP-2 as a prognostic indicator for gastric carcinoma patients. The notion that MMP-2 is a valuable indicator of gastric cancer progression and prognosis is supported by immunohistochemical, zymography and mRNA studies showing that MMP-2 is associated with tumour invasion, lymph node metastasis and survival (Allgayer *et al*, 1998; Mönig *et al*, 2001; Chuanzhong *et al*, 2002; Kabashima *et al*, 2002; Liu *et al*, 2002a; Elnemr *et al*, 2003; Yokoyama *et al*, 2004; Ji *et al*, 2005). The value of MMP-2 as an independent prognostic marker for gastric carcinomas is underscored by our observation that MMP-9, MMP-7, MMP-8, TIMP-1 and TIMP-2 have no prognostic relevance.

Matrix metalloproteinase-9 levels were enhanced in some clinicopathological subgroups of gastric cancer, that is, according to the Laurén classification and for WHO differentiation grade. The association between MMP-9 and early stages of gastric carcinoma, as shown before (Torii *et al*, 1998; Kabashima *et al*, 2000), was also present in our study. In contrast to our previous findings, high MMP-9 levels did not show a significant correlation with survival and also not for the ratio MMP-9/TIMP-1 (data not shown) as recently suggested (Zhang *et al*, 2003). One obvious explanation for the discrepancy with our previous data is the small number of patients in the study.

However, the relatively high MMP-9 levels in early gastric carcinomas also might affect the relation between MMP-9 and prognosis, especially in our extended follow-up study using tumour-related survival.

Matrix metalloproteinase-7, MMP-8, TIMP-1 and TIMP-2 were included in the present study as comparisons to evaluate the prognostic strength of MMP-2 and MMP-9. Matrix metalloproteinase-7 was selected because MMP-7 production in various types of carcinomas has predominantly been found in tumour cells and because MMP-7 was recently suggested as potential marker for gastric carcinoma (Liu *et al*, 2002b). Although enhanced levels were found in the different carcinoma subgroups, for example, TNM stage and Laurén's intestinal type, there was no correlation between high MMP-7 levels and patients survival. This contrasts in part with several other studies reporting not only a clear association between MMP-7 expression and gastric cancer progression but also with survival (Liu *et al*, 2002b; Ajisaka *et al*, 2004). Essential differences with our study are, however, that the latter studies were carried out using immunohistochemistry, focusing on MMP-7-expressing carcinoma cells at the invasive front, whereas our ELISA antigen values were derived from representative overall parts of the tumours. Matrix metalloproteinase-8, like MMP-9, is mainly present in neutrophils in carcinomas. Therefore, the expected correlation in presence of MMP-8 and MMP-9 was confirmed by the high correlation between both antigen levels (ρ 0.810, $P \leq 0.001$, $n = 158$), and the similar distribution according to the different cancer subgroups. The lack of correlation with survival was, therefore, not surprising in this study, as described by others before (Yokoyama *et al*, 2004).

The levels of TIMP-1 were significantly enhanced in cancer tissue, but the previously found association between TIMP-1 levels in sections or homogenates from gastric cancer tissue with survival (Joo *et al*, 2000; Yoshikawa *et al*, 2001) was not observed in our group of patients. However, our group contained relatively less patients with advanced TNM stages, which could account for the different results compared with these former studies. In contrast to what was expected from *in vitro* studies (Koyama, 2004), we did not find differences between TIMP-2 levels in normal and cancerous tissue. Also, the levels between different tumour subgroups did not vary, indicating a rather constitutive expression of this inhibitor. As TIMP-2 immunohistochemical staining combined with *in situ* hybridisation experiments detected the expression of TIMP-2 in gastric cancer tissue, primarily in peritumoral stromal cells rather than in malignant cells (Joo *et al*, 2000), we conclude that the localisation of TIMP-2 within the cancerous tissue might be of crucial importance but apparently not the total amount of the inhibitor. The recently suggested role for TIMP-2 in the activation of pro-MMP-2 (Itoh *et al*, 2001), combined with the different cell types involved in the expression of MMP-2 and its main inhibitor TIMP-2 in gastric carcinoma, indicate the importance of local cell –cell and molecule –molecule interactions in the activation process. This is

particularly noticeable from our finding that there is no correlation between MMP-2 and TIMP-2 levels in the tissue homogenates, where the increase in MMP-2 outbalances that of TIMP-2, resulting in an increased net MMP-2 activity in the tumours, an observation which can only be made by using the BIA. This process was not observed with MMP-9 and TIMP-1, where a more balanced increase was found in the tumours. Although many *in vitro* studies, animal models and clinical studies clearly showed that MMPs are indeed involved in a number of critical steps during tumour growth and invasion, most synthetic MMP inhibitors, designed as anticancer agents, failed to improve patients outcome in clinical trials (Zucker *et al*, 2000), showing that our understanding of the working mechanisms of MMPs in tumour biology is still poor. Coincidentally, gastric cancer appeared to be one of the few cancers for which a significant survival benefit from therapy with a matrix metalloproteinase inhibitor has been described (Bramhall *et al*, 2002). Recent studies indicate that proteolytic MMP activity is involved in the uncovering or release of specific sites from macromolecules in the extracellular matrix (McCawley and Matrisian, 2001; Polette *et al*, 2004), which at least *in vitro* leads to various biological activities. Our study shows that an enhanced MMP-2 level is consistently and more strongly associated with prognosis of gastric cancer patients than other MMPs or TIMPs. This association might be caused by the noninvasion-related activities of MMPs, like cytokine release/activation, which makes MMP-2 in our opinion an important player in gastric cancer, deserving further investigation. Finally, differences in the association of the other MMPs and TIMPs with gastric cancer survival between our study and other reports, as mentioned above, might be related to differences in genetic background, that is, Caucasian vs Asian, which is currently under study.

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CHAPTER 6

Clinical evidence for a protective role of lipocalin-2 against MMP-9 autodegradation and the impact for gastric cancer

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Abstract

Recently, complexes of matrix metalloproteinase MMP-9 with lipocalin-2 (neutrophil gelatinase-associated lipocalin) were found in the urine obtained from breast cancer patients, while these were completely absent in that obtained from healthy controls. *In vitro* data suggested a possible role for lipocalin-2 in the protection of MMP-9 against autolysis.

To establish this effect *in vivo*, we determined the presence of MMP-9, lipocalin-2 and their complex in tumour tissue from 81 gastric cancer patients. The effect of the presence of the individual parameters, the complexes, and the inhibitors TIMP-1 and TIMP-2 on MMP-9 activity was evaluated with a bioactivity assay. Immunohistochemical (double) staining identified epithelial cells as the most likely cellular source. Finally, evaluation of all these parameters with clinicopathological scores revealed that tumour MMP-9/lipocalin-2 complexes were significantly related with the classifications of Laurén and WHO, and highly associated with worse survival in Cox's univariate (HR 2.087, P=0.006) and multivariate analysis (HR 2.095, P=0.025).

Introduction

Lipocalin-2 (also known as neutrophil gelatinase-associated lipocalin) is a member of the highly heterogeneous family of lipocalins, sharing a common tertiary structure [1, 2]. Lipocalin-2 has initially been discovered in specific granules of human neutrophils [3] and was later shown to be expressed also by certain epithelial cells, in particular during inflammatory or cancerous circumstances [4-10]. There is little information about the physiological functions of lipocalins, but lipocalin-2 has been associated with cellular iron uptake, antibacterial activity, and epithelial cell differentiation [2, 9].

Enhanced tissue, blood and urine levels of matrix metalloproteinase-9 (MMP-9) have been associated with the malignancy of various tumour types [11-14]. Using quantitative zymography and immunoassays we have previously shown that MMP-9 as well as MMP-2 are enhanced in gastric cancer tissue and that high levels are associated with worse survival of the patients [15, 16]. Next to MMP-9 and MMP-2, the zymograms revealed extra bands, particularly between 125-135 kDa. These bands have been described before in the urine obtained from cancer patients, and are most likely complexes of MMP-9 with lipocalin [17, 18]. *In vitro* experiments suggested a role for lipocalin-2 in the protection of MMP-9 against autolysis [17].

To investigate the suggested relevance of MMP-9/lipocalin-2 complexes *in vivo*, we determined the levels of MMP-9, lipocalin-2 and their complex in tissue homogenates from 81 gastric carcinomas in comparison with adjacent normal mucosa from the same patients. We used immunohistochemical staining of paraffin-embedded tissue sections to establish the cellular origin of MMP-9 and lipocalin-2. To confirm the histological findings, the levels of MMP-9, lipocalin-2 and the MMP-9/lipocalin-2 complexes in the homogenates were compared with markers for neutrophils, a known source of MMP-9 and lipocalin-2. The effect of complex formation between MMP-9 and lipocalin-2 on the MMP-9 activity state was evaluated using a specific MMP-9 bioactivity assay. Finally, the possible clinical consequence of the presence of MMP-9/lipocalin-2 complexes in gastric tumours was evaluated by examining for correlations with established clinicopathological parameters of the carcinoma patients, including univariate and multivariate Cox proportional hazard survival analyses.

Materials & methods

Patients and study design

Fresh tissue specimens from 81 patients (21 females and 60 males, mean age 65.9 years, range 35.1-91.3) who underwent resection for primary gastric adenocarcinoma between 1984 and 1996 at the department of Oncologic Surgery, Leiden University Medical Centre were collected prospectively. Samples from the mid-central non-necrotic part of the carcinoma and from normal mucosa, taken approximately 10 cm from the tumour, were snap-frozen and stored at –70°C until extraction. All carcinomas were classified according to the TNM classification (UICC 1992), and localization as well as diameter of the tumour was registered. Microscopical histological parameters, including differentiation-grade, WHO-, Borrmann-, and Laurén-classification, as well as the presence of intestinal metaplasia in the normal gastric mucosa, were revised by a gastroenterologist and a pathologist. All patients entered the study at operation date, and the patient's time experience ended in the event of death or, when still alive, at the common closing date. The minimal follow-up was 33 months with a decreasing overall survival according to TNM stage, i.e. from TNM I (52.2%, n=23), to TNM II (26.9%, n=26), to TNM III (28%, n=25), and to TNM IV (0%, n=7). The study was performed according to the instructions and guidelines of the LUMC medical ethics committee.

Tissue preparation and protein concentration

Homogenisation of tissue specimens and determination of protein concentrations were performed as described previously [15].

MMP-9/lipocalin-2 complex zymography

Quantitative gelatin zymography for MMP-9/lipocalin-2 complexes was performed as described before [15], using an Ultroscan XL Laser Densitometer (LKB) for quantification. The MMP-9/lipocalin-2 complex levels in tissue homogenates were expressed in arbitrary units (AU) per mg protein.

ELISAs for MMP-9, lipocalin-2, MMP-9/lipocalin-2-complexes, MMP-8 and TIMPs

Total antigen levels of MMP-9, lipocalin-2, and MMP-8 were determined using previously described ELISAs [19-22]. The concentrations of MMP-9/lipocalin-2 complexes, TIMP-1 and TIMP-2 were measured using commercial ELISAs according to the manufacturer instructions (R&D Systems Europe, Abingdon, UK). The MMP-9/lipocalin-2 ELISA immobilizes complexes via anti-MMP-9 antibodies followed by detection using anti-lipocalin-2 antibodies and does not detect MMP-9 or lipocalin-2 in their free forms.

MMP-9 activity assay

The bioactivity assay (BIAs) for MMP-9 was done as described previously [14, 19, 22]. This assay detects active MMP-9 and total MMP-9 levels in parallel in 96-wells plates coated with MMP-9 specific antibodies and using modified MMP-sensitive pro-urokinase as substrate. The fraction of the latent MMP-9 proform is calculated by subtraction of active from total MMP-9.

Myeloperoxidase (MPO) activity assay

MPO activity was measured as described previously [23]. In short, tissue homogenates were incubated with 0.5% hexadecyl-trimethylammonium bromide in 50 mM potassium phosphate buffer (pH 5.5), plus 0.026% *ortho*-dianisidine dihydrochloride substrate and 0.018% H_2O_2. The reaction kinetics were followed for 30 min at 450 nm in 96-well plates. The specificity of the reaction was checked with sodium azide (0.1 mM). All samples were analyzed in duplicate and standardized using a homogenate of pooled human neutrophils, and MPO activity was expressed in arbitrary units.

Immunohistochemistry and immunofluorescence double staining

Paraffin sections (5 µm) from the same tumours as used for the homogenates were deparaffinized and stained for the localisation of MMP-9 and lipocalin-2. Antigen retrieval was performed through boiling in a 0.01 M citrate solution (pH 6.0) for 12 minutes in a microwave oven. After being rinsed in PBS and incubated with 10 % of normal goat serum (Dako) for 30 minutes, the sections were incubated with the primary antibody polyclonal rabbit anti-lipocalin-2 (1:100, from Drs H. Tschesche and O. Hiller) or polyclonal rabbit anti-MMP-9 (1:400, TNO, Leiden, The Netherlands) overnight at 4 degrees. After washing, the sections were incubated with biotinylated goat anti-rabbit 1:400 (Dako) for 30 minutes, followed by washing and incubation with Streptavidin/ABCcomplex/HRP (DakoCytomation) for 30 minutes. The brown colour was developed by 0.004 % H_2O_2 (Merck) and 0.05 % diaminobenzidine tetrahydrochloride (Sigma) in 0.01 M Tris-HCl pH 6.0 for 10 minutes. The slides were counterstained with Mayer's haematoxylin (Merck). For specific cell recognition, i.e. epithelial cells, (myo)fibroblasts, neutrophils and endothelial cells, sequential tissue sections were stained with mouse anti-pan-cytokeratin (1:1000, clone C11, Santa Cruz biotechnologies, Santa Cruz, USA), mouse anti-vimentin (1:400, clone V9 Santa Cruz), mouse anti-smooth muscle actin (1:1000, clone ASM-1, Progen Heidelberg, Germany), rabbit anti-myeloperoxidase (1:1000, Dako) and mouse anti-CD31 (1:400, clone JC70A, Dako) followed by appropriate second antibodies and staining procedures. Immunofluorescence double staining was performed as described before [24]. In short, sections were incubated for 1 hr with rabbit polyclonal anti-lipocalin-2 and mouse monoclonal anti-MMP-9 (clone GE-213, 1:400, NeoMarkers, Fremont, CA) antibodies, appropriately

diluted in PBS with 1% BSA, washed, and incubated with respectively Alexa Fluor 488- and 546-conjugated anti-rabbit and anti-mouse antibodies (Molecular Probes, Leiden, The Netherlands) diluted in PBS-BSA. After incubation and washing, the sections were mounted in Mowiol. A Zeiss LSM 510 confocal microscope equipped with argon and He/Ne lasers and a 20x objective were used to obtain the images.

Statistical analysis

Differences between normal and tumour values for all parameters were calculated using the Wilcoxon signed ranks test and visualized by Box-Whisker graphs using lower and upper margins of 5%. Correlations between parameters were determined according to Spearman's Rho test. For the survival analyses the clinicopathological parameters were dichotomized as described previously [15], unless indicated. Cut off values for MMPs and related factors were optimised. Survival analyses were performed with the Cox proportional hazards model using the SPSS Windows Release 12.0.1. Statistical Package (2004, SPSS Inc., Chicago, Illinois, USA). Multivariate survival analyses were performed using the Cox proportional hazards method by separately adding

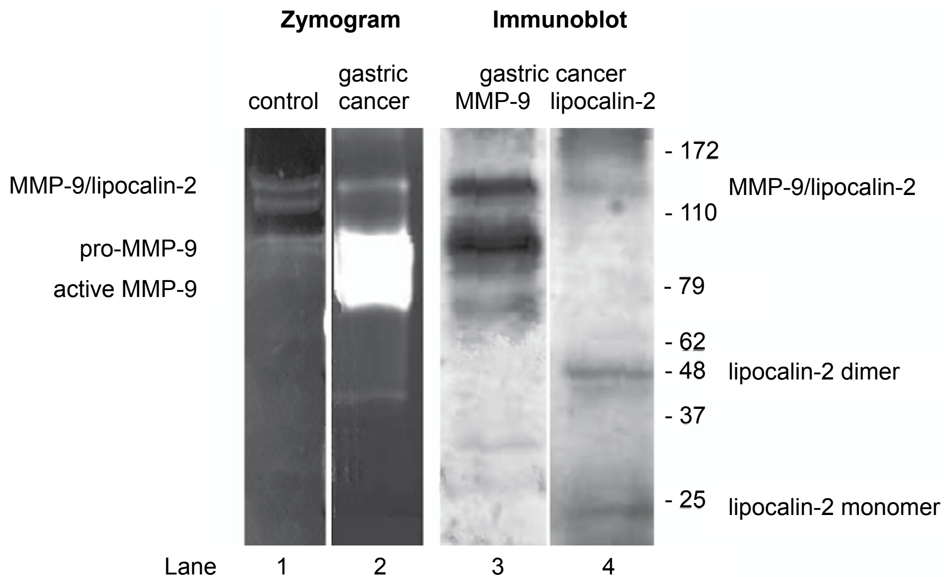

Figure 1. Zymogram and immunoblot showing MMP-9, MMP-9/lipocalin-2 complexes and lipocalin-2 in a representative gastric cancer tissue homogenate (lane 2-4). MMP-9 activity is located in the zymograms (lane 2) between 70-92 kDa, representing active MMP-9 and pro-MMP-9, and at 135 kDa corresponding with MMP-9/lipocalin-2 complex standard (lane 1). The immunoblots show corresponding complex bands for MMP-9 (lane 3) and lipocalin-2 (lane 4) with extra bands at approximately 25 and 50 kDA representing respectively the monomer and homodimer forms of lipocalin-2. Lane 1 contains 20 µl standard from the MMP-9/lipocalin-2 ELISA (≈ 0.8 ng).

the significant MMP variables to the dichotomized clinicopathological parameters. Survival curves were constructed using the method of Kaplan and Meier including the Log-rank test. Differences were considered significant when $P \leq 0.05$.

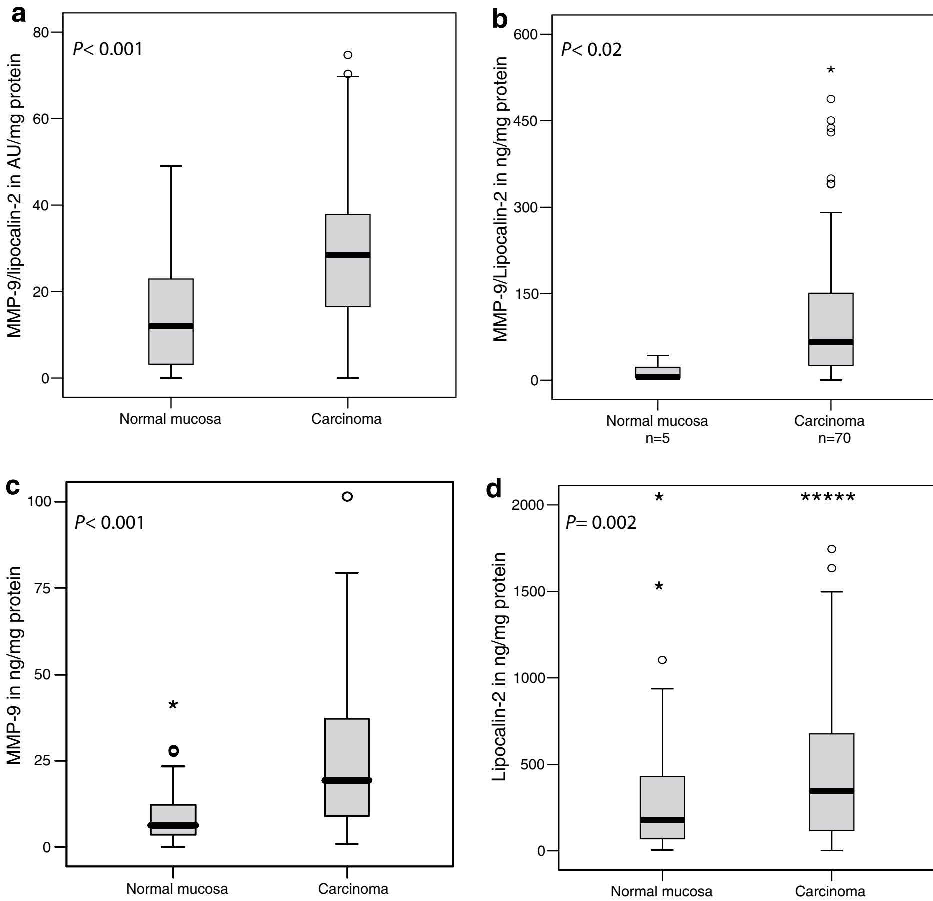

Figure 2. Levels of a) MMP-9/lipocalin-2 complex in AU/mg protein, b) MMP-9/lipocalin-2 complex in ng/mg protein, c) MMP-9 in ng/mg protein, and d) lipocalin-2 in ng/mg protein in carcinoma tissue and adjacent normal mucosa from 81 gastric cancer patients. *n*=81 unless indicated.

Results

Quantification of MMP-9/lipocalin-2 complexes in gastric cancer tissue homogenates

The presence of MMP-9/lipocalin-2 complexes in tissue homogenates from gastric cancer patients was determined using zymography and ELISA. Figure 1 shows a typical gastric cancer homogenate with in the zymogram abundant MMP-9 mediated lysis and a smaller band at molecular weight 135 kDa, corresponding with standard MMP-9/lipocalin-2 complex. The nature of this band was further verified using immunoblots for respectively MMP-9 and lipocalin-2 under normal (Figure 1) and reduced conditions (not shown). The amount of the MMP-9/lipocalin-2 complexes was quantified from the zymograms, using laser densitometry (Figure 2a). MMP-9/lipocalin-2 complexes were significantly enhanced in cancer tissue compared with control mucosa (27.3±2.0 versus 14.5±1.4 AU/mg protein, $P<0.001$, $n=81$). The data from this semi-quantitative assay were compared with the results obtained with a commercial ELISA (Figure 2b). The correlation between both assays was highly significant (rho = 0.488, $P<0.0001$, $n=75$, i.e. 5 normal mucosa and 70 carcinoma homogenates).

Levels of MMP-9 and lipocalin-2 in gastric cancer tissue homogenates

The tissue levels of MMP-9 and lipocalin-2 are shown in figure 2c and d. The gastric carcinomas contained significant higher concentrations of MMP-9 ($P<0.001$) and

Table 1 - Correlation coefficients (ρ plus *P*-values) for MMP-9, lipocalin-2 and MMP-9/lipocalin-2 complexes in relation to myeloperoxidase (MPO), MMP-8 and TIMP-1 in 162 gastric cancer tissue homogenates (81 normal/81 cancer).

	MMP-9	Lipoc-2	MMP-9/Lipocalin-2 complex	MMP-9 active	MMP-9 latent
MMP-9 ng/mg protein		0.438 (0.000)	0.641 (0.000)	0.240 (0.003)	0.817 (0.000)
Lipocalin-2 ng/mg protein			0.273 (0.001)	-0.121 ns	0.443 (0.000)
MMP-9/Lipoc-2 AU/mg protein				0.166 (0.038)	0.586 (0.000)
MMP-9 active U/mg protein					0.263 (0.001)
MPO AU/mg protein	0.486 (0.000)	0.280 (0.000)	0.332 (0.000)	0.073 (ns)	0.462 (0.000)
MMP-8 ng/mg protein	0.810 (0.000)	0.482 (0.000)	0.578 (0.000)	0.128 ns	0.734 (0.000)
TIMP-1 ng/mg protein	0.358 (0.000)	0.363 (0.000)	0.315 (0.000)	-0.097 (ns)	0.240 (0.004)

lipocalin-2 (P=0.002) than adjacent normal tissues. In general, lipocalin-2 was more abundantly present than MMP-9, in specific cases even more than 100 times higher.

Correlation between MMP-9 and MMP-9/lipocalin-2 with MMP-9 activity state

The correlation of MMP-9, lipocalin-2, and MMP-9/lipocalin-2-complex with MMP-9 activity in tissue homogenates of gastric cancer patients is shown in Table 1. Active MMP-9 levels correlated significantly with the total antigen level of MMP-9, but more interestingly also with the MMP-9/lipocalin-2 concentration (P=0.038), suggesting a protective role for lipocalin-2-complex formation in MMP-9 (auto)activation. The tis-

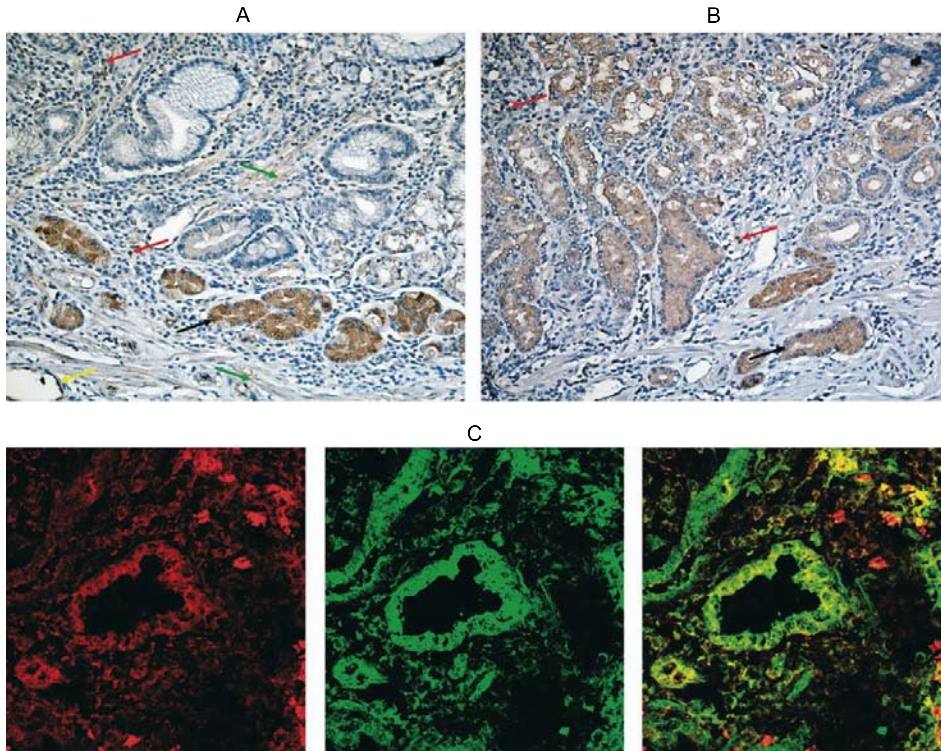

Figure 3. Typical immunohistochemical staining of a human gastric intestinal type carcinoma for: a) MMP-9 (200x) and b) lipocalin-2 (200x). Black, red, green and yellow arrows indicate, respectively, epithelial cells, neutrophil-like cells, (myo)fibroblast like cells and endothelial cells. Protein levels in corresponding homogenate for MMP-9, lipocalin-2 and complex are respectively 29 ng/mg, 4928 ng/mg and 17 AU/mg protein. c) Immunofluorescence double staining (400x) for MMP-9 (red) and Lipocalin-2 (green). Yellow colour suggests complex formation.

sue concentration of TIMP-1, the most relevant tissue inhibitor of MMP-9, was equally correlated with the levels of MMP-9 and lipocalin-2, but not with MMP-9 activity.

Immunohistochemical staining for MMP-9 and lipocalin-2

To establish the cellular source of the MMP-9/lipocalin-2 complexes, sequential paraffin sections adjacent to the tissue used for homogenates were stained for MMP-9 and lipocalin-2. Normal mucosa showed barely any staining for MMP-9 nor lipocalin-2 (not shown). In carcinoma tissues staining for MMP-9 was found in neutrophils and a substantial part of the epithelial cells, occasionally in endothelial cells, and incidentally in muscle cells, macrophages, and fibroblasts (Figure 3a). In neutrophils and epithelial cells lipocalin-2 was similarly distributed compared with MMP-9, but lipocalin-2 was additionally present in tumour epithelial subgroups which lacked MMP-9 staining (Figure 3b). Endothelial cells and fibroblasts showed little or no staining for lipocalin-2. Immunofluorescence double staining confirmed that particular epithelial cells stained for lipocalin-2 but not for MMP-9 (Figure 3c red versus green). Furthermore this staining revealed that only a fraction of MMP-9 and lipocalin-2 was actually in close proximity (Figure 3c, yellow versus green). Yellow staining was found in particular at the perifery of cells, suggesting that the majority of both proteins is uncomplexed and presumably still compartmentalized within the cells, as suggested by zymographic analysis.

Correlations between MMP-9, lipocalin-2, MMP-9/lipocalin-2, MMP-8 and MPO

To confirm the similarities and the apparent difference between MMP-9 and lipocalin-2 in cellular origin, as found by immunohistochemistry, the concentrations of MMP-9, lipocalin-2 and MMP-9/lipocalin-2-complex in the tissue homogenates were evaluated for correlations with the levels of MPO and MMP-8 (Table 1). MPO, a commonly used cell marker for neutrophils, correlated strongly with MMP-8, a collagenase abundantly present in neutrophils (0.445, $P<0.0005$) as well as with MMP-9, but the correlation with lipocalin-2 was considerably less, suggesting a possible other source of lipocalin-2 than neutrophils only.

Relation between MMP-9/lipocalin-2 complexes and clinicopathological parameters

The MMP-9/lipocalin-2 levels were significantly enhanced in differentiated tumours according to the WHO classification (30.9±2.5 vs. 19.6±2.8 AU/mg protein, $P\leq0.006$) and in tumours of the intestinal type (30.5±2.6 vs. 21.9±2.7 AU/mg protein, $P\leq0.04$). MMP-9/lipocalin-2 levels showed a trend to increase with higher TNM stages. Dichotomization of the patients, based on low (AU<36) or high (AU>36) MMP-9/lipocalin-2 complex values in their tumour, showed a significant correlation with overall survival

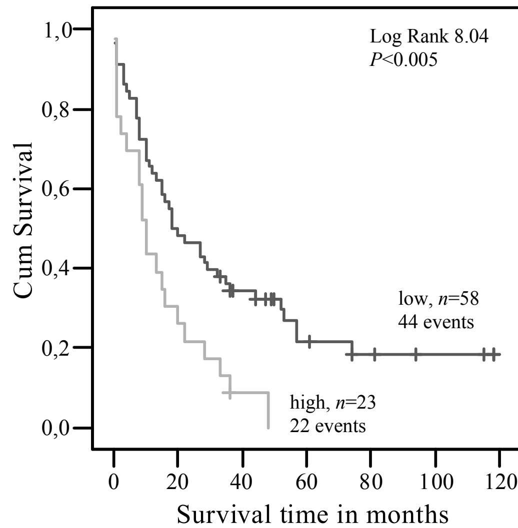

Figure 4. Kaplan-Meier survival curve for a cohort of gastric cancer patients subdivided by low (≤36 AU/mg protein) or high (>36 AU/mg protein) levels of MMP-9/lipocalin-2 complex in their tumour tissue homogenate.

Table 2 - Univariate and multivariate Cox proportional hazard overall survival analyses for low or high levels of MMP-9/lipocalin-2 in tissue homogenates of gastric cancer *versus* different clinicopathological parameters.

			Univariate			Multivariate		
		n	HR	CI 95%	*P*	HR	CI 95%	*P*
Gender	F/M	21/60	1.247	0.730-2.131	NS	1.622	0.900-2.923	NS
Age	<median>	40/41	1.323	0.815-2.149	NS	1.504	0.860-2.629	NS
TNM	1	23/81	1	-	-	1	-	-
	2	26/81	1.984	1.033-3.813	0.040	2.133	1.009-4.639	0.047
	3	25/81	1.586	0.804-3.130	NS	1.623	0.737-3.704	NS
	4	7/81	3.248	1.261-8.366	0.015	6.027	1.876-20.46	0.003
Laurén	dif/mx vs inte	30/50	1.103	0.671-1.816	NS	1.125	0.402-3.137	NS
WHO	diff vs undiff	54/26	0.881	0.525-1.480	NS	0.874	0.289-2.609	NS
Borrmann	fung. vs infiltr.	55/24	1.025	0.591-1.778	NS	0.846	0.457-1.567	NS
Localization	cardia vs rest	36/45	0.603	0.368-0.989	0.045	0.419	0.223-0.764	0.005
Diameter	≤5 vs >5 cm	47/34	1.062	0.652-1.729	NS	0.695	0.403-1.195	NS
Eosinophils	few vs many	56/24	1.220	0.725-2.053	NS	1.846	1.023-3.544	0.042
Intest. metaplasia	not vs present	39/42	0.551	0.334-0.909	0.020	0.651	0.365-1.151	NS
MMP-9 antigen	<median>	40/40	1.143	0.701-1.863	NS	1.336	0.756-2.363	NS
Lipocalin-2	<median>	40/39	1.029	0.632-1.674	NS	0.772	0.422-1.413	NS
MMP-9/lipocalin-2	≤36 vs >36 AU	58/23	2.087	1.229-3.544	0.006	2.095	1.099-4.031	0.025

NS: non significant

(Log Rank 8.04, *P*<0.005, *n*=81), as shown in figure 4. Analysis of the MMP-9/lipocalin-2 complex ELISA data showed a similar trend but did not reach statistical significance (Log Rank, 3.04, *P*=0.0815, *n*=70).

Survival analyses

The relation of MMP-9/lipocalin-2 complexes with survival was further characterized with Cox's uni- and multivariate analyses against the clinicopathological parameters (Table 2). The level of MMP-9/lipocalin-2 was significantly associated with worse survival and kept its significance in multivariate analyses, indicating its value as an independent prognostic factor.

Discussion

High levels of lipocalin-2 have been reported in various types of cancer [6-10]. Our study shows that lipocalin-2 levels are indeed significantly enhanced in gastric carcinomas compared to adjacent control tissue. Moreover and more interestingly, our data show that the complexes of lipocalin-2 with MMP-9 are also significantly enhanced in human gastric tumours.

In vitro experiments showed that lipocalin-2 is able to induce the expression of E-cadherin, to promote the formation of polarized epithelia, and to diminish the invasiveness and metastasis of Ras-transformed cells [25], suggesting a protective role against cancer. Other studies reported a positive correlation between lipocalin-2 expression levels and the growth rate of lipocalin-2 transfected MCF-7 human breast carcinoma cells, which were subcutaneously implanted in immuno-deficient mice [18]. Immunohistochemical analyses of these xenografted tumours showed that the over-expression of lipocalin-2 was accompanied by enhanced levels of MMP-9, suggesting the formation of complexes between MMP-9 and lipocalin-2. The formation of MMP-9/lipocalin-2 complexes has previously been shown to protect MMP-9 from auto-degradation *in vitro* [17, 18]. MMP-9/lipocalin-2 complex formation could result in increased extracellular, tumour-associated MMP-9, and hence in enhanced tumour growth as recently suggested by Fernández et al. [18]. We found that in gastric cancer tissue lipocalin-2 levels are in general 30 times higher than corresponding MMP-9 levels, presumably leading to MMP-9/lipocalin-2 complex formation of a substantial part of the MMP-9 fraction after it has been released from the cells. These complexes were significantly correlated with the active, as well as the latent fraction of MMP-9. Therefore, our data support the hypothesis that enhanced production of lipocalin-2 in cancerous tissue stimulates the formation of a complex with MMP-9, playing a role in the maintenance of an extracellular pool of a latent form of this powerful proteinase,

by prevention from auto-degradation. This latent pool of secreted, lipocalin-2-bound MMP-9 has previously been shown to be important for the spatial control of VEGF release from the ECM and hence for enhanced angiogenesis [26]. Our study does not provide information about the presence and/or role of MMP-9/lipocalin-2/TIMP-1 complexes. These ternary complexes have previously been isolated from phorbol myristate acetate stimulated neutrophils and showed low gelatinase activity, as expected [27]. In our study, total TIMP-1 levels correlated significantly with all the forms of MMP-9, except for the active form of MMP-9, suggesting that other factors are involved in regulating the activity of MMP-9, besides the ratio between MMP-9 and TIMP-1. TIMP-2 levels were weakly inversely correlated with MMP-9 antigen levels, suggesting little or no mutual interaction (data not shown).

The quantitative determination of MMP-9 and lipocalin-2 in tissue homogenates, as performed in this study, has several advantages compared to semi-quantitative immunohistological detection methods but obviously does not provide information about the localization of the proteins. Our immunohistochemical data revealed that lipocalin-2 as well as MMP-9 in gastric cancers are mainly present in neutrophils and epithelial cells, but that epithelial expression of MMP-9 is depending on the individual cancer and on the location within the tumour. MMP-9 was furthermore found in (myo) fibroblast-like cells and endothelial cells. These data are in accordance with what has been found previously in colonic cancer [6, 28]. Our fluorescent double-staining data suggest that, although MMP-9 and lipocalin-2 seem present in close proximity especially within the cells, overlap of green and red colours, presumably representing extra-cellular complex formation, is limited and mainly restricted to peri-cellular areas. Whether the enhancement of MMP-9/lipocalin-2 complexes in gastric cancer compared with adjacent normal mucosa was caused by the influx of neutrophils or alternatively by upregulated expression in malignant epithelial cells, could not be established in this study. The finding that high numbers of intra-tumoural neutrophils are associated with better survival of patients with gastric cancer [29], would suggest the latter.

From this study, the clinical relevance of MMP-9/lipocalin-2 complex formation appears most obvious from the correlation with overall survival of the patients. Enhanced levels of these complexes were highly prognostic for worse survival, whereas the levels of single MMP-9 and lipocalin-2 were not. The finding that MMP-9/lipocalin-2 levels are increased in gastric cancer tissue and that enhancement might be associated with clinical outcome of the patients is supported by a recent study reporting that similar complexes were present in approximately 90% of the urines obtained from breast cancer patients, but not in those from healthy controls [18]. The prognostic value of MMP-9/lipocalin-2 complexes is in accordance with the presumed role of lipocalin-2 in the protection of secreted MMP-9 against auto-degradation, which contributes to

an enhanced pool of potentially active MMP-9, a proteolytic enzyme associated with angiogenesis and tumour growth. High total MMP-9 levels were not associated with survival in the present study. This is not in agreement with what we have published previously [15], but those earlier data were based on a smaller group of patients and on detection of MMP-9 activity instead of total antigen level. The different outcome between both studies indicates the delicacy of the use of proteinase levels as prognostic indicators, as discussed before [16, 30]. Apparently not just the enhanced presence, but more the (potential) activation state of the proteinase, i.e. the result of, respectively, production, release, activation, and the inactivation by inhibitors, seems to be crucial, similar to what has been described for other enzyms playing a role in gastric cancer like urokinase and MMP-2 [16, 31]. Additionally, our data indicate that prevention of auto-degradation of MMP-9 by lipocalin-2 might play an important role too.

In conclusion, we have shown for the first time that complexes between MMP-9 and lipocalin-2 are present in enhanced levels in gastric cancer tissue and that high levels are associated with worse survival of the patients. The potential clinical value of our findings should be confirmed in larger groups of cancer patients. Recently the enzymatic activity of MMP-9/lipocalin-2 complex has indeed been found to correlate significantly with the depth of tumour invasion in esophageal squamous cell carcinomas [32].

Acknowledgements

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Conflict of interest

None declared

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CHAPTER 7

Clinical impact of MMP and TIMP gene polymorphisms in gastric cancer

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Abstract

Gastric cancers express enhanced levels of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Single-nucleotide polymorphisms (SNPs) in MMP and TIMP genes may be associated with disease susceptibility and might also affect their antigen expression. We studied the genotype distribution and allele frequencies of SNPs of MMP-2, -7, -8 and -9 and TIMP-1 and -2 in gastric cancer patients in relation to tumour progression, patient survival and tissue antigen expression. The genotype distribution and allele frequencies were similar in gastric cancer patients and controls, except for MMP-7$_{-181A>G}$. In addition, the genotype distribution of MMP-7$_{-181A>G}$ was associated with *Helicobacter pylori* status (χ^2 7.8, $P = 0.005$) and tumour-related survival of the patients. Single-nucleotide polymorphism TIMP-2$_{303C>T}$ correlated significantly with the WHO classification (χ^2 5.9, $P = 0.03$) and also strongly with tumour-related survival (log rank 11.74, $P = 0.0006$). Single-nucleotide polymorphisms of MMP-2, -8, -9 and TIMP-1 were not associated with tumour-related survival. Only the gene promoter MMP-2$_{-1306C>T}$ polymorphism correlated significantly with the protein level within the tumours. First-order dendrogram cluster analysis combined with Cox analysis identified the MMP-7$_{-181A>G}$ and TIMP-2$_{303C>T}$ polymorphism combination to have a major impact on patients survival outcome. We conclude that MMP-related SNPs, especially MMP-7$_{-181A>G}$ and TIMP-2$_{303C>T}$, may be helpful in identifying gastric cancer patients with a poor clinical outcome.

Introduction

In the process of tumour dissemination and metastasis, matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play an important role in the invasion of tissue, vascular and lymphatic basal membranes and the subsequent coordinated proteolytic breakdown and reconstitution of extracellular matrix (Kohn and Liotta, 1995). Matrix metalloproteinases also modulate cell proliferation, apoptosis and host immune surveillance (Egeblad and Werb, 2002). Immunohistochemical and *in situ* hybridisation studies as well as quantitative assays have demonstrated that gastric carcinomas contain enhanced amounts of MMPs (Nomura *et al*, 1995; Honda *et al*, 1996; Mori *et al*, 1997). We previously reported significantly enhanced MMP and TIMP levels in gastric carcinomas, but only MMP-2 was independently associated with a poor overall survival of the patients (Kubben *et al*, 2006). Single-nucleotide polymorphisms (SNPs) within MMP genes are thought to influence the expression of MMPs and/or even seem to be associated with the susceptibility for the development of malignancy. For instance, a functional SNP in the MMP-2 gene promoter (-1306C>T) was found to be associated with the risk of the development, but not the metastatic behaviour of gastric cardia adenocarcinoma, in an ethnic Chinese population (Miao *et al*, 2003). Furthermore, the frequency of a functional SNP of MMP-7 (-181A>G) was found to be significantly higher in gastric cardiac carcinoma patients compared to controls in another Chinese study (Zhang *et al*, 2005). Particularly, genotypes with the MMP-7_{-181G} allele (A/G + G/G) showed a significantly increased susceptibility for gastric cardiac carcinoma with an odds ratio of 1.96 (Zhang *et al*, 2005). Finally, a significant association in Japanese gastric cancer patients was found between an SNP in the promoter of the MMP-9 gene (-1562C>T) and the degree of tumour invasion, clinical stage and lymphatic invasion (Matsumura *et al*, 2005). However, as indicated above, these studies on MMP-SNPs in gastric carcinoma patients describe ethnic Chinese and Japanese populations with a known high incidence of gastric cancer.

In the present study, we determined the genotype distribution and allele frequencies of SNPs of MMP-2, -7, -8 and -9, and of TIMP-1 and -2 in a cohort of 79 Caucasian gastric carcinoma patients, in which we previously assessed clinical relevance of the respective protein levels. In order to get insight into the functional and clinical contribution of these MMP-related gene polymorphisms, we assessed the relation between the distribution of these SNPs and the respective protein levels in tumour and adjacent normal tissue as well as the relation of the SNPs with established clinicopathological parameters and the relation of the gene polymorphisms with tumour-related survival.

Materials and methods

Patients and study design

Fresh histologically normal tissue specimens of 79 patients (21 females and 58 males, mean age 66 years, range 35 –91 years) who underwent resection for primary gastric adenocarcinoma at the department of Oncologic Surgery of the Leiden University Medical Centre were collected prospectively, as described before (Janssen *et al*, 2002). Various clinicopathological data were (re-)evaluated or collected from patient files by one gastroenterologist and one pathologist (Janssen *et al*, 2002). All carcinomas were classified according to the TNM classification (Hermanek and Sobin, 1992) and localisation as well as diameters of the tumours were registered. Microscopical histological parameters, including differentiation-grade, classification according to WHO, Borrmann and Laurén, as well as the presence of *Helicobacter pylori (Hp)* and intestinal metaplasia in the normal gastric mucosa were assessed. All patients entered the study at operation date and a patient's time experience ended in the event of death or, when still alive, at the common closing date. The minimal follow-up was 33 months with a decreasing overall survival according to TNM stage, that is, from TNM I (n = 23), to TNM II (n = 24), to TNM III (n = 25), and to TNM IV (n = 7). Genomic DNA was isolated using the salting out method (Miller *et al*, 1988). In addition, DNA was extracted from peripheral blood leucocytes of 169 healthy volunteers (38% male, median age 33 years (range 18 –73 years), >95% Caucasian) as described before (van der Veek *et al*, 2005).

Single-nucleotide polymorphism analyses

Genotypes were analysed by PCR-based techniques as described in Table 1.

Antigen determination and protein concentration

From 50–100mg of wet tissue samples, homogenates were prepared. The samples were wet weighted, and 1 ml of 0.1 M Tris-HCl (pH 7.5) with 0.1% ($v.v^{-1}$) Tween-80 extraction buffer per 60 mg sample was added as described previously. The protein concentration was determined using the method of Lowry *et al* (1951). Specific ELISAs for the MMP and TIMP antigen determination were performed as recently described (Kubben *et al*, 2006).

Statistical analysis

Statistical analyses were performed using SPSS11.0 Statistical Package (2004, SPSS Inc., Chicago, IL, USA). Hardy –Weinberg analysis was performed using the chi-square (χ^2) or Fisher's exact test to examine differences in the distribution of alleles and genotypes between patients and controls. Odds ratios and confidence intervals

Table 1 - Primer sequences and PCR conditions for amplification of MMP and TIMP SNPs

SNP	Method	Primer	Sequence	Location	Annealing	BP	Enzyme	Reference
MMP-2$_{-1575G>A}$	RFLP-PCR	Outer primers	ACCAGACAAGCCTGAACTTGTCTGA TGTGACAACCGTCTCTGAGGAATG	Promoter	63°C, 35 cycles	542	*Bsp*HI	(Harendza *et al*, 2003)
MMP-2$_{-1306C>T}$	Tetra-primer ARMS-PCR	Outer forward	ACCAGACAAGCCTGAACTTGTCTGA	Promoter	63°C, 35 cycles	542		(Ye *et al*, 2001)
		Outer reverse	TGTGACAACCGTCTCTGAGGAATG			3792		
		Inner forward	ATATTCCCCACCCAGCACGCT			11		
		Inner reverse	GCTGAGACCTGAAGAGCTAAAGAGTTG					
MMP-7$_{-181A>G}$	RFLP-PCR	Forward Reverse mismatch	TGGTACCATAATGTCCTGAATG TCGTTATTGGCAGGAAGCACACAATGAATT	Promoter	55°C, 35 cycles	150	*Eco*RI	(Jormsjö *et al*, 2001)
MMP-7$_{-153C>G}$	RFLP-PCR	Forward mismatch	ACGAATACATTGTGTGCTTCCTGCCAATCA	Promoter	55°C, 30 cycles	158	*Nla*III	(Jormsjö *et al*, 2001)
		Reverse	TTTATATAGCTTCTCAGCCTCG					
MMP-8$_{-799C>T}$	RFLP-PCR	Forward Reverse	CTGTTGAAGGCCTAGAGCTGCTGCTCC CATCTTCTCTTCAAACTCTACCC	Promoter	58°C, 35 cycles	968	*Sfc*I	(Wang *et al*, 2004)
MMP-8$_{+17C>G}$	RFLP-PCR	Forward	CTGTTGAAGGCCTAGAGCTGCTGCTCC	Transcription start	58°C, 35 cycles	668	*Dde*I	(Wang *et al*, 2004)
		Reverse	CATCTTCTCTTCAAACTCTACCC					
MMP-9$_{-1562C>T}$	RFLP-PCR	Forward	ATGGCTCATGCCCGTAATC	Promoter	60°C, 38 cycles	352	*Nla*III or *Sph*I	(Zhang *et al*, 1999)
		Reverse	TCACCTTCTTCAAAGCCCTATT					
TIMP-1$_{372C>T}$	RFLP-PCR	Forward	GCACATCACTACCTGCAGTC	Exon 5 phe 124 phe	54°C, 35 cycles	175	*Bss*SI	(Wollmer *et al*, 2002)
		Reverse	GAAACAAGCCCACGATTTAG					
TIMP-2$_{-418G>C}$	RFLP-PCR	Forward Reverse	CGTCTCTTGTTGGCTGGTCA CCTTCAGCTCGACTCTGGAG	Promotor	64°C, 35 cycles	304	*Bso*BI	(Zhou *et al*, 2004)
TIMP-2$_{303C>T}$	RFLP-PCR	Forward	TAGGAACAGCCCCACTTCTG	Exon 3 ser 101 ser	60°C, 35 cycles	119	*Tsp*RI	(Krex *et al*, 2003)
		Reverse	CCTCCTCGGCAGTGTGTG					

ARMS = amplification refractory mutation system; MMP = matrix metalloproteinase; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; SNP = single-nucleotide polymorphism; TIMP = tissue inhibitor of metalloproteinase. Deliberate mismatches in primers are underlined.

(95%) were calculated by logistic regression. For the tumour-related survival analysis, the clinicopathological parameters were dichotomised as described before (Sier *et al*, 1996). Univariate survival analyses were performed with the Cox proportional hazards model, using the clinicopathological parameters and MMP-SNPs, resulting in the identification of covariates that significantly correlated with the survival of the patients. Multivariate survival analysis was performed by separately adding the MMP-SNPs variables to all the dichotomised clinicopathological parameters. Tumour-related survival curves were constructed using the method of Kaplan and Meier including the log rank test. Group means for antigen levels were compared using two-tailed Mann–Whitney U-tests. Differences were considered significant when $P \leq 0.05$.

Results

The genotype distribution and allele frequencies of the SNPs for MMP-2, -7, -8, -9, TIMP-1 and -2 for the 79 gastric cancer patients and 169 control subjects are summarised in Table 2. Single-nucleotide polymorphisms 1306C>T and 1575G>A for MMP-2 were found to be in complete linkage disequilibrium and consequently, in the rest of the study only MMP-2$_{-1306C>T}$ will be described. None of the genotype distributions in the control group or in the cancer patients deviated from the Hardy–Weinberg equilibrium (data not shown). Matrix metalloproteinase-7$_{-181A>G}$ was the only polymorphism differently distributed among gastric carcinoma patients compared with control subjects: AA 43.0%, AG 46.8%, and GG 10.1% in patients *vs* AA 27.2%, AG 62.7% and GG 10.1% in controls ($P<0.04$; Table 2). Comparison of the genotype distribution of our Caucasian control subjects with those published on other mainly Asiatic control groups (Wollmer *et al*, 2002; Ghilardi *et al*, 2003; Krex *et al*, 2003; Miao *et al*, 2003; Wang *et al*, 2004; Zhou *et al*, 2004; Matsumura *et al*, 2005; Zhang *et al*, 2005) showed significant differences for MMP-2$_{-1306C>T}$, MMP7$_{-181A>G}$, TIMP-1$_{372C>T}$ and TIMP-2$_{-418G>C}$ (Table 3).

All the SNPs were evaluated for association with the clinicopathological parameters. Correlations were found for MMP2$_{-1306C>T}$ with Borrmann's classification (fungating *vs* infiltrating: CC 70% and CT/TT 30% *vs* CC 48% and CT/TT 52%; χ^2 3.5, $P = 0.06$), MMP-7$_{-181A>G}$ with the presence of *Hp* (negative *vs* positive: AA 60% and AG/GG 40% *vs* AA 21% and AG/GG 79%; χ^2 7.8, $P = 0.005$) and TIMP-2$_{303C>T}$ with the WHO classification (differentiated *vs* not differentiated: CC 93% and CT/TT 7% *vs* CC 72 and CT/TT 28%; χ^2 5.9, $P = 0.03$).

The prognostic value for tumour-related survival of the respective SNPs was analysed using Cox proportional hazards analyses (Table 4). In the univariate analyses, TIMP-2$_{303C>T}$ was significantly correlated with survival (Figure 1A), whereas MMP7$_{-181A>G}$

Table 2 - Allele frequencies and genotype distribution of MMP and TIMP SNPs in gastric carcinoma patients ($n = 79$) and controls ($n = 169$)

SNP			Patients								Controls												
			n	%		*n*	%		*n*	%		*n*	%		*n*	%		*n*	%	χ^2	*P*	OR	CI
MMP-2$_{-1306C>T}$	Allele	C	124	78.5				T	34	21.5	C	257	76.0				T	81	24.0	0.362	NS		
	Genotype	CC*	50	63.3	CT	24	30.4	TT	5	6.3	CC	102	60.4	CT	53	31.4	TT	14	8.3	0.361	NS	0.833	0.51 – 1.53
MMP-7$_{-181A>G}$	Allele	A	105	66.5				G	53	33.5	A	198	58.6				G	140	41.4	2.810	NS		
	Genotype	AA*	34	43.0	AG	37	46.8	GG	8	10.1	AA	46	27.2	AG	106	62.7	GG	17	10.1	6.533	<0.04	0.495	0.28 – 0.87
MMP-7$_{-153C>T}$	Allele	C	149	94.3				T	9	5.7	C	320	94.7				T	18	5.3	0.029	NS		
	Genotype	CC*	70	88.6	CT	9	11.4	TT	—	0	CC	151	89.3	CT	18	10.7	TT	—	0	0.031	NS	1.079	0.46 – 2.52
MMP-8$_{-799C>T}$	Allele	C	84	53.2				T	74	46.8	C	191	56.5				T	147	43.5	0.487	NS		
	Genotype	CC*	19	24.1	CT	46	58.2	TT	14	17.7	CC	55	32.5	CT	81	48.0	TT	33	19.5	2.509	NS	1.524	0.83 – 2.80
MMP-8$_{+17C>G}$	Allele	C	147	93.0				G	11	7.0	C	309	91.4				G	29	8.6	0.380	NS		
	Genotype	CC*	68	86.1	CG	11	13.9	GG	—	0	CC	141	83.4	CG	27	16.0	GG	1	0.6	0.660	NS	0.781	0.37 – 1.66
MMP-9$_{-1562C>T}$	Allele	C	137	86.7				T	21	13.3	C	286	84.6				T	52	15.4	0.376	NS		
	Genotype	CC*	59	74.7	CT	19	24.0	TT	1	1.3	CC	120	71.0	CT	46	27.2	TT	3	1.8	0.394	NS	0.830	0.45 – 1.52
TIMP-1$_{372C>T}$	Allele	C	74	46.8				T	84	53.2	C	167	49.4				T	171	50.6	0.285	NS		
	Genotype ♀	CC*	5	23.8	CT	10	47.6	TT	6	28.6	CC	24	22.4	CT	59	55.2	TT	24	22.4	0.481	NS	0.925	0.31 – 2.79
	♂	C*	27	46.6				T	31	53.4	C	30	48.4				T	32	51.6	0.040	NS	1.076	0.53 – 2.21
TIMP-2$_{303C>T}$	Allele	C	146	92.4				T	12	7.6	C	301	89.0				T	37	11.0	1.359	NS		
	Genotype	CC*	68	86.1	CT	10	12.7	TT	1	1.3	CC	133	78.7	CT	35	20.7	TT	1	0.6	2.588	NS	0.598	0.29 – 1.25
TIMP-2$_{-418G>C}$	Allele	G	157	99.4				C	1	0.6	G	337	99.7				C	1	0.3	0.305	NS		
	Genotype	GG*	78	98.7	GC	1	1.3	CC	—	0	GG	168	99.4	GC	1	0.6	CC	—	0	0.306	NS	2.154	0.13 – 34.9

CI = confidence interval; MMP = matrix metalloproteinase; NS = not significant; OR = odds ratio; PCR = polymerase chain reaction; SNP = single-nucleotide polymorphism; TIMP = tissue inhibitor of metalloproteinase. The χ^2 test was used to examine differences in the distributions of alleles and genotypes between patients and controls. OR and 95% CI were calculated by logistic regression using marked genotypes (*) as reference groups.

Table 3 - Comparison of genotype distributions of the control subjects from this study ($n = 169$, 107♀/62♂) with the control groups from previously published studies

MMP-2 −1306C>T	MMP-7 −181A>G	MMP-7 −153C>T	MMP-8 −799C>T	MMP-8 +17C>G	MMP-9 −1562C>T	TIMP-1 372C>T	TIMP-2 303C>T	TIMP-2 −418G>C
Lin *et al*, 2004 $n = 147$ (A) χ^2 6.0 NS	Ghilardi *et al*, 2003 $n = 111$ (C) χ^2 1.7 NS	Ghilardi *et al*, 2003 $n = 111$ (C) χ^2 1.7 NS	Wang *et al*, 2004 $n = 216$ (B) χ^2 3.8* NS	Wang *et al*, 2004 $n = 216$ (B) χ^2 0.1* NS	Demacq *et al*, 2006 $n = 200$ (♂C) χ^2 5.8 NS	Krex *et al*, 2003 $n = 24$♀/20♂ (C) ♀χ^2 4.1, ♂χ^2 5.0 NS, $P \leqslant 0.025$	Krex *et al*, 2003 $n = 41$ (C) χ^2 0.3 NS	Hirano *et al*, 2001 $n = 40$ (A) χ^2 66.6 $P \leqslant 0.001$
Miao *et al*, 2003 $n = 789$ (A) χ^2 16.7 $P \leqslant 0.001$	Zhang *et al*, 2005 $n = 350$ (A) χ^2 217.2 $P \leqslant 0.001$				Lose *et al*, 2005 $n = 392$ (C) χ^2 0.7 NS	Lose *et al*, 2005 $n = 34$♀/33♂ (C) ♀χ^2 8.2, ♂χ^2 1.3 NS, NS	Wang *et al*, 1999 $n = 82$ (C) χ^2 3.7* NS	Zhou *et al*, 2004 $n = 509$ (A) χ^2 66.7 $P \leqslant 0.001$
(Xu *et al*, 2004) $n = 126$ (A) χ^2 8.6 $P \leqslant 0.025$					Matsumura *et al*, 2005 $n = 224$ (A) χ^2 0.2 NS	Wollmer *et al*, 2002 $n = 159$♀/114♂ (C) ♀χ^2 8.0, ♂χ^2 0.0 $P \leqslant 0.025$, NS		
Zhou *et al*, 2004 $n = 509$ (A) χ^2 23.1 $P \leqslant 0.001$								

MMP = matrix metalloproteinase; NS = not significant; TIMP = tissue inhibitor of metalloproteinase. *Allele distribution. (A): Asiatic population, (B): Afro-American population, (C): Caucasian population.

Table 4 - Univariate and multivariate Cox proportional hazard analysis for gastric cancer patients testing SNPs for MMP and TIMP *vs* clinicopathological parameters

			Univariate			Multivariate		
Parameter		***n***	**HR**	**CI 95%**	***P***	**HR**	**CI 95%**	***P***
Gender	F *vs* M	21–58	0.706	0.390–1.278	NS	0.606	0.322–1.138	NS
Age	<median>	40–39	1.231	0.709–2.138	NS	1.422	0.749–2.701	NS
TNM	1	23	1	—	—	1	—	—
	vs 2	24	3.041	1.302–7.102	0.01	4.282	1.629–11.257	0.003
	vs 3	25	2.995	1.293–6.933	0.01	3.119	1.175–8.280	0.022
	vs 4	7	7.175	2.420–21.271	0.0005	19.661	5.096–75.855	0.0005
Laurén	diffuse/mix *vs* intestinal	28–50	0.913	0.522–1.595	NS	1.281	0.344–4.774	NS
WHO	differentiated *vs* undiff.	53–25	1.152	0.652–2.033	NS	1.846	0.470–7.251	NS
Borrmann	fungating *vs* infiltrating	54–23	1.077	0.576–2.013	NS	0.677	0.338–1.356	NS
Localisation	Rest *vs* cardia	45–34	1.715	0.980–3.001	0.059	2.878	1.410–5.874	0.004
Diameter	$\leqslant 5$ *vs* >5 cm	45–34	1.07	0.615–1.861	NS	0.612	0.324–1.158	NS
Intestinal metaplasia	Not *vs* present	37–42	0.499	0.283–0.880	0.016	0.704	0.378–1.312	NS
SNP								
MMP-$2_{-1306C>T}$	CC *vs* CT/TT	50–29	0.756	0.421–1.358	NS	1.158	0.578–2.321	NS
MMP-$7_{-181A>G}$	AA *vs* AG/GG	34–45	1.718	0.965–3.057	0.066	1.637	0.850–3.152	NS
MMP-$7_{-153C>T}$	CC *vs* CT	70–9	1.096	0.467–2.575	NS	1.137	0.396–3.269	NS
MMP-$8_{-799C>T}$	CC *vs* CT/TT	19–60	0.681	0.376–1.234	NS	0.607	0.302–1.222	NS
MMP-$8_{+17C>G}$	CC *vs* CG	68–11	1.349	0.656–2.775	NS	1.364	0.516–3.606	NS
MMP-$9_{-1562C>T}$	CC *vs* CT/TT	59–20	1.127	0.598–2.126	NS	1.006	0.482–2.101	NS
TIMP-$1_{372C>T}$	CC *vs* CT/TT	32–47	1.125	0.644–1.967	NS	0.739	0.387–1.411	NS
TIMP-$2_{303C>T}$	CC vs CT/TT	68–11	3.224	1.571–6.616	0.001	4.445	1.808–10.928	0.001
TIMP-$2_{-418G>C}$	GG *vs* GC	78–1	ND	ND	ND	ND	ND	ND
MMP-$7_{-181A>G}$ and	AA-CC	31	1	—	—	1	—	—
TIMP-$2_{303C>T}$	*vs* AG/GG-CC	37	1.896	1.011–3.558	0.046	1.911	0.947–3.856	0.071
	vs AA or AG/GG-CT/TT	11	3.859	1.578–9.442	0.003	5.323	1.736–16322	0.003

CI = confidence interval; F = female; HR = hazard ratio; M = male; MMP = matrix metalloproteinase; ND = not defined; NS = not significant; SNP = single-nucleotide polymorphism; TIMP = tissue inhibitor of metalloproteinase; TNM = tumour node metastasis; WHO = World Health Organisation.

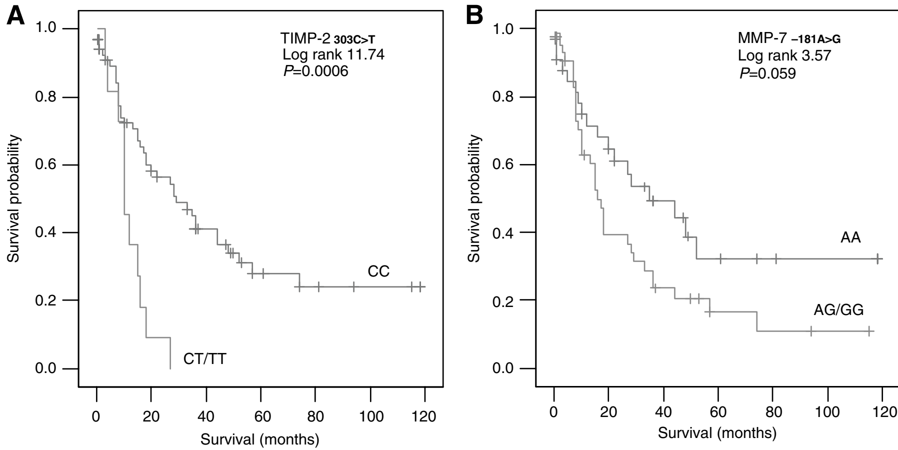

Figure 1. Survival curves using tumour-related death for 79 gastric cancer patients subdivided by the presence of a SNP in (A) the TIMP-2 gene (303C>T) and (B) the MMP-7 gene (-181A>G).

showed a trend (Figure 1B). From the clinicopathological parameters, only TNM classification and the presence of intestinal metaplasia were significantly associated with survival, whereas the localisation showed a trend. In a multivariate analysis against all the clinical parameters $TIMP\text{-}2_{303C>T}$ kept its significance, indicating its potential value as an independent prognostic marker. A dendrogram showing a two-dimensional unsupervised hierarchical cluster analysis for all 79 patients using all the SNPs determined in this study is presented in Figure 2. Interestingly, the first-order cluster (I) separated the eight patients with mutations in both the survival-associated SNPs, that is, $MMP\text{-}7_{-181A>G}$ and $TIMP\text{-}2_{303C>T}$, from the rest of the patients. Further analyses of this SNP combination revealed a stepwise and statistically significant poorer tumour-related survival for these mutations (0% (0 out of 11 patients) *vs* 32% (12 out of 37 patients) *vs* 52% (16 out of 31 patients); χ^2 9.7, $P \leq 0.01$). Cox analyses confirmed this prognostic significance of this $MMP\text{-}7_{-181A>G}$– $TIMP\text{-}2_{303C>T}$ combination, as indicated in Table 4 and illustrated in Figure 3.

The relation between the genotype distribution of the SNPs and the protein levels in normal and tumour tissue is shown in Table 5. As expected, the exon-located SNPs were not found to be accompanied by changes in the respective protein levels. The promoter-located SNPs showed some trends with the protein levels, but the only relevant significant difference was found for $MMP\text{-}2_{-1306C>T}$ within tumour tissue.

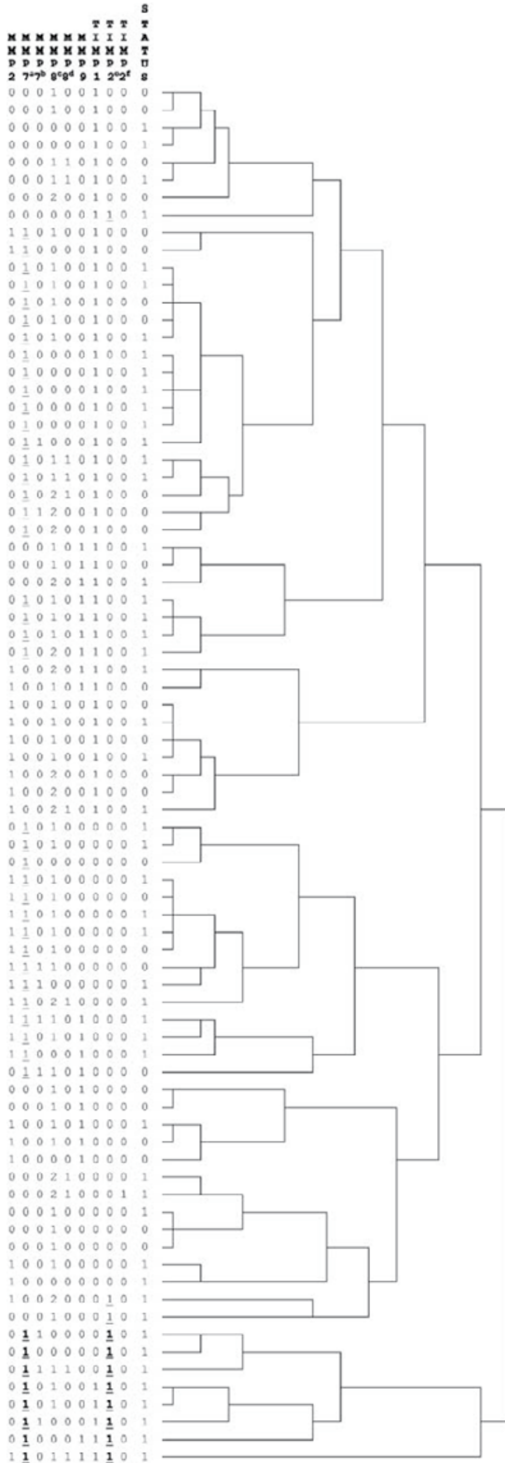

Figure 2. Dendrogram of a two-dimensional unsupervised hierarchical cluster analysis for 79 gastric cancer patients using SNPs of MMP2$_{-1306C>T}$, MMP-7$_{-181A>C}$(A), $_{-153C>G}$(B), MMP-8$_{-799C>T}$(C), $_{+17C>G}$(D), MMP-9$_{-1562C>T}$, TIMP-$_{1372C>T}$, and TIMP-2$_{303C>T}$(E), $_{-418G>C}$(F). For all the SNPs, 0 stands for the reference genotype and 1 for the combined other genotypes as described in Table 2. Because of the distribution, for MMP-8$_{-799C>T}$(C) a three-group subdivision was used: 0 =CC, 1 =CT, 2 =TT. Status: 0 =alive or not tumour-related death, 1 = tumour-related death.

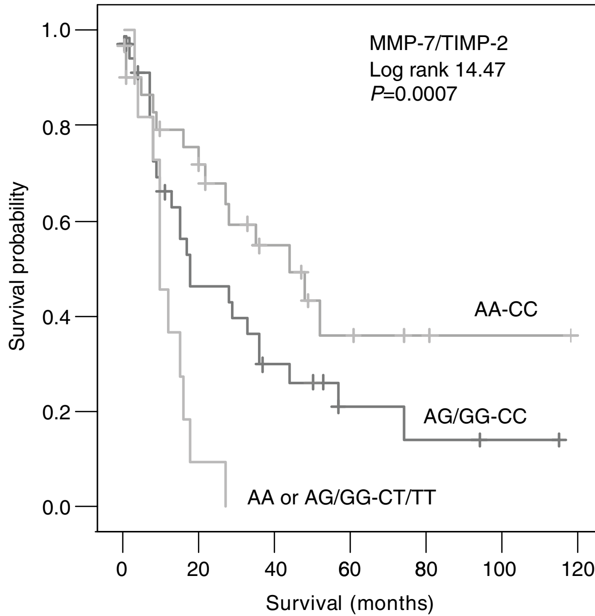

Figure 3. Survival curves using tumour-related death for 79 gastric cancer patients subdivided by the presence of combined polymorphisms in the MMP-7 gene (-181A>G) and TIMP-2 gene (303C>T).

Discussion

Because some gene polymorphisms of MMPs and TIMPs have been found to be related to disease susceptibility and changed gene transcription *in vitro*, we investigated whether gastric cancer is associated with SNPs of MMP-2, -7, -8 and -9, or their inhibitors TIMP-1 and TIMP-2. The only SNP that was distributed significantly differently among gastric carcinoma patients compared to our control population was MMP-$7_{-181A>G}$, with more patients of the AA genotype than in controls. The latter was not expected from previous studies on gastrointestinal cancer (Ghilardi *et al*, 2003; Zhang *et al*, 2005) and is most likely caused by ethnic differences (Asiatic *vs* Caucasian; Table 3), disease localisation (gastric *vs* colon) and the relatively low number of patients included in the studies. In our study, the gastric cancer patients with the variant AG/GG genotype showed worse survival data than the AA patients (Table 4 and Figure 1B), although the difference did not fully reach statistical significance. The fact that tumours of the AG/GG patients did not contain higher MMP-7 antigen levels in our study suggests that the presence of SNP $MMP7_{-181A>G}$ alone is not directly translated into an enhanced tumour MMP-7 antigen expression or activity. However, considering the previously shown localised presence of MMP-7 at the invasive front of tumours, immunohistochemical or *in vitro* studies might further elucidate this

Table 5 - Association between the presence of SNPs and the protein levels (mean ± s.e.m. in ng mg-1 protein) within tissue of MMPs and TIMPs in 79 gastric carcinoma patients

SNP		Protein level					
Located in promoter		**Normal mucosa**		***P*-value**	**Tumour**		***P*-value**
MMP-$2_{-1306C>T}$	CC *vs* CT/TT	5.0 ± 0.5	4.5 ± 0.7	NS	18.2 ± 2.4	14.9 ± 3.8	0.03
MMP-$7_{-153C>T}$	CC *vs* CT[a]	2.2 ± 0.6	0.7 ± 0.0	0.019	47.1 ± 14.1	46.1 ± 16.4	NS
MMP-$7_{-181A>G}$	AA *vs* AG/GG	1.3 ± 0.4	2.1 ± 0.6	NS	52.1 ± 22.3	43.4 ± 15.0	NS
MMP-$8_{-799C>T}$	CC *vs* CT/TT	139 ± 31	83 ± 12	0.044	305 ± 67	326 ± 60	NS
MMP-$8_{+17C>G}$	CC *vs* CG	98 ± 19	95 ± 15	NS	302 ± 51	440 ± 140	NS
MMP-$9_{-1562C>T}$	CC *vs* CT/TT	9.7 ± 1.1	7.0 ± 1.5	NS	26.9 ± 2.8	19.4 ± 3.3	NS
TIMP-$2_{-418G>C}$	GG *vs* GC[b]	6.0 ± 0.3	5.1	NS	6.3 ± 0.4	5.2	NS
Located in exon							
TIMP-$1_{372C>T}$	CC *vs* CT/TT	8.7 ± 1.6	7.7 ± 0.7	NS	18.8 ± 2.6	15.7 ± 1.4	NS
TIMP-$2_{303C>T}$	CC *vs* CT/TT	6.0 ± 0.3	5.6 ± 0.6	NS	6.0 ± 0.4	7.5 ± 1.6	NS

MMP = matrix metalloproteinase; NS = not significant; SNP = single-nucleotide polymorphism; TIMP = tissue inhibitor of metalloproteinase. [a] $n = 3$. [b] $n = 1$.

functional relationship. The other striking correlation of MMP-7$_{-181A>G}$ in this study is with the presence of *Hp*. Gastric cancer patients with the AG/GG genotype were significantly more often *Hp*-positive, which might indicate an enhanced susceptibility for this bacterium. The presence of *Hp* is associated with the development of gastric cancer and stimulation of MMP-7 production by *Hp* in human gastric epithelial cells has previously been suggested as a possible mechanism predisposing towards gastric neoplasia (Wroblewski *et al*, 2003; Chen *et al*, 2004).

Tissue inhibitor of metalloproteinase-2 is involved in the regulation of MMP-2 activity (Howard *et al*, 1991; Wang *et al,* 2000). In addition, TIMP-2 has been shown to promote cell growth (Hayakawa *et al*, 1994). Enhanced amounts of TIMP-2 protein are found to be associated with prostate cancer malignancies (Ross *et al*, 2003), but for colon and gastric cancer the correlation with clinicopathological parameters has not been established (Ring *et al*, 1997; Joo *et al*, 2000). In our study, the CT/TT variant of TIMP-2303C4T was observed more frequently in undifferentiated gastric carcinomas (WHO classification) and it was associated with worse tumour-related survival of gastric cancer patients. Tissue inhibitor of metalloproteinase-2$_{303C>T}$ is located in exon 3 with no effect on the final amino-acid sequence of the protein (S101S) and no effect on the total TIMP-2 expression between gastric normal and tumour tissue (Table 5). Therefore, the TIMP-2$_{303C>T}$ SNP behaves as a disease susceptibility gene polymorphism by a so far undefined mechanism. The other SNP for TIMP-2 in this study (-418G>C), localised in the promoter of the gene, has been described to abolish the Sp1-binding site and therefore may downregulate TIMP-2 gene expression (Hirano *et al*, 2001). A previous study reported that the variant TIMP-2$_{-418G>C}$ genotype (GC or CC) was indeed associated with a moderately reduced risk of breast cancer in a Chinese population (Zhou *et al*, 2004). Because our group of Caucasian gastric cancer patients contained only one patient with the variant genotype (GC), we could not determine an association with tumour staging, patient survival or antigen expression.

The first-order cluster in a two-dimensional unsupervised hierarchical cluster analysis including all SNPs clearly separated the patients with mutations in both the survival-associated SNPs, that is, MMP-7$_{-181A>G}$ and TIMP-2$_{303C>T}$ from the rest of the patients. Cox analysis confirmed this SNP combination as a prognostic parameter for gastric cancer. Although results of cluster analysis of SNPs in gastric cancer have not been published before, hierarchical cluster analysis of patterns of chromosomal aberrations in gastric cancer patients identified patients with worse prognosis as well (Weiss *et al*, 2003), confirming the validity of such an approach.

The (-1306C>T) SNP in the promoter of the MMP-2 gene has also been found to diminish promoter activity by abolishing the Sp1-binding site (Price *et al*, 2001). Consequently, the variant genotypes (CT/TT) are expected to produce less MMP-2 antigen, which consequently might be associated with decreased cancer risk or better

survival of the patients (Sier *et al*, 1996). Although we did not find a significant difference in distribution of MMP-2$_{-1306C>T}$ between gastric cancer patients and controls, the tumours from patients with the CT/TT genotypes contained significantly less MMP-2 antigen than the CC genotype (Table 5). This relation was expected, but as far as we know, never shown before. The fact that the MMP-2$_{-1306C>T}$ status on its own was not correlated with survival might be explained by the complicated activation mechanism of MMP-2 in which several other proteins are involved. Changes in MMP-2 antigen levels are therefore not directly correlated with MMP-2 activity levels. The fact that we did not find a relation with survival in our group of patients supports the study of Miao *et al* (2003), describing that the CC genotype was not associated with higher risk of metastasis at the time of diagnosis. A weak but significant difference in genotype distribution of MMP-2$_{-1306C>T}$ and gastric carcinomas, classified according to the Bormann classification, was observed with the highest percentage of the CC genotype in type 1/2 (fungating) preceding infiltrating tumours (type 3/4). This underscores the role of MMP-2 in breaking down the extracellular matrix in early gastric cancer which has been suggested before (Miao *et al*, 2003).

The genotype distribution of MMP-9$_{-1562C>T}$ in our group of healthy controls was not different from other publications. We did not find differences in genotype distribution for MMP-9$_{-1562C>T}$ between gastric cancer patients and controls either, which is in agreement with the study of Matsumura *et al* (2005) in Japanese patients. However, that study showed significant associations of the CT/TT genotype with depth of invasion, lymphatic invasion and TNM classification. In our study, MMP-9$_{-1562C>T}$ was not correlated with clinicopathological parameters or survival. Moreover, MMP-9 antigen levels in normal as well as tumour tissue of gastric cancer patients with the MMP-9$_{-1562C>T}$ genotype were not enhanced, as was recently also found in plasma of healthy subjects (Demacq *et al*, 2006). Our results indicate that the presence of the T allele variant in the MMP-9 promoter ($_{-1562C>T}$) is not associated with clinical outcome in our Caucasian group of gastric cancer patients.

Neutrophils secrete both gelatinase B (MMP-9) and neutrophil collagenase (MMP-8) after stimulation. Matrix metalloproteinase-8 expression levels correlated with tumour stage and poor prognosis in ovarian cancer (Stadlmann *et al*, 2003). Levels of MMP-8 and -9 correlated significantly with each other and with TIMP-1 levels, but were not related to tumour size or prognosis in human breast cancer (Duffy *et al*, 1995). Nothing has been published thus far about SNPs for MMP-8 and cancer, but three MMP-8 promoter haplotypes (MMP-8$_{-799C>T}$, MMP-8$_{+17C>G}$ and MMP-8$_{-381A>G}$) have been found to be associated with preterm rupture of membranes in delivery, indicating a functional role on MMP-8 expression (Wang *et al*, 2004). Because MMP-8$_{+17C>G}$ and MMP8$_{-381A>G}$ were found to be in complete linkage disequilibrium, we decided to study the distribution of MMP-8 $_{-799C>T}$, MMP8$_{+17C>G}$ in our group of gastric cancer patients. However,

we did not find any relation of both SNPs with protein levels, clinicopathological parameters, or survival in this study.

TIMP-1 is a ubiquitous glycoprotein capable of inhibiting all activated collagenases (Gomez *et al*, 1997). Tissue inhibitor of metalloproteinases were previously found not to be correlated with tumour stage, histological type, lymph node status or survival in human gastric cancer (Murray *et al*, 1998). We did not find any relation of TIMP-$1_{372C>T}$ with gastric carcinoma, protein level or survival of the patients.

Taken together, our data indicate that MMP and TIMP gene polymorphisms contribute to gastric carcinogenesis. Determination of these gene polymorphisms, especially MMP-$7_{-181A>G}$ and TIMP-$2_{303C>T}$ both as single parameter and in combination as a cluster, might be helpful to identify gastric cancer patients with a poor clinical outcome and in need of (neo)-adjuvant treatment aiming at better outcome.

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CHAPTER 8

Summarizing discussion

Introduction

The studies presented and discussed in this thesis focus on the clinical relevance of matrix metalloproteinases (MMPs) in gastric inflammation and cancer. Because *Helicobacter pylori* gastritis is associated with gastric cancer [1], studies on the involvement of the gelatinases MMP-2 and MMP-9 in *H. pylori*-induced gastritis are described, including the influence of eradication therapy. Next, studies regarding the clinical impact of MMPs, tissue inhibitors of metalloproteinases (TIMPs) and neutrophil-gelatinase associated lipocalin (NGAL) in gastric cancer are addressed. As single-nucleotide polymorphisms (SNPs) within MMP and TIMP genes may be associated with disease susceptibility and altered antigen expression, subsequently the genotype distribution of SNPs of MMPs and TIMPs in gastric cancer was studied, and their relation with established clinicopathological parameters including survival. Finally, the concept of MMP inhibition as a potential treatment modality for gastric inflammation and cancer is discussed.

Matrix metalloproteinases in gastrointestinal inflammation and carcinogenesis

A brief review about the role and function of MMPs, TIMPs and lipocalins in gastrointestinal inflammation and carcinogenesis is provided in **chapter 1**. MMPs are a family of zinc-dependent proteinases that play an important role in destruction and repair of the extracellular matrix and basement membranes in various physiological and pathological processes including gastrointestinal inflammation and carcinogenesis. Depending on their structure and substrate preference, the MMP family is divided into collagenases, stromelysins, matrilysins, gelatinases, elastases and membrane-type MT-MMPs [2]. The studies in this thesis focus on MMP-2, MMP-7, MMP-8 and MMP-9 because of their presumed clinical relevance in gastric cancer. Gelatinase-A (MMP-2) and gelatinase-B (MMP-9) are gelatinases that can specifically degrade basement membrane type IV and gelatins. MMP-2 is predominantly produced by stromal cells, whereas MMP-9 is predominantly secreted by inflammatory cells, especially neutrophils [3]. Neutrophil collagenase (MMP-8) is one of the collagenases that is synthesized exclusively by neutrophils before emigration from the bone marrow into the peripheral circulation. Matrilysin (MMP-7) is predominantly expressed by epithelial or tumour cells and can activate pro-MMP-9 [4, 5]. TIMPs are naturally occurring tissue inhibitors of metalloproteinases that can form inhibitory complexes with most MMPs. TIMP-1 predominantly binds to pro-MMP-9 and TIMP-2 to pro-MMP-2 [6]. Lipocalins are a group of small extracellular proteins that are involved in various biological pro-

cesses including the regulation of cell homeostasis, the modulation of the immune response and, as carrier proteins, act in the clearance of endogenous and exogenous substances. Neutrophil gelatinase-associated lipocalin (NGAL, lipocalin-2) is stored in secondary granules of neutrophils and can form heterodimers with neutrophil gelatinase-B (MMP-9) [7].

The gelatinases MMP-2 and MMP-9 in *Helicobacter pylori*-induced gastritis

The results of an investigation whether gastric mucosal MMP-2 and MMP-9 levels were affected by *H. pylori* infection in 45 patients with *H. pylori*-induced gastritis compared to 27 *H. pylori* negative control patients are presented in **chapter 2**. In patients with *H. pylori*-induced gastritis, significantly increased MMP-9 levels in both antrum and corpus mucosa were found, with a good correlation with the severity of the mucosal inflammation. The increase of MMP-9 in the corpus mucosa of patients with antral gastritis only was intermediate to that of *H. pylori* negative control patients and of patients with pangastritis. Immunohistochemically, MMP-9 was predominantly observed in inflammatory and stromal cells, and in zymogen-producing chief cells of corpus mucosa. In contrast, MMP-2 levels were almost unaltered when compared with *H. pylori* negative patients.

As *H. pylori*-induced gastritis is associated with gastric malignancy and *H. pylori*-induced gastritis and gastric carcinoma are accompanied by alterations in the MMP levels, subsequently a study was performed to investigate whether *H. pylori*-affected gastric mucosal MMP-2 and MMP-9 levels were reversible after successful eradication therapy (**chapter 3**). Therefore, 58 patients with *H. pylori*-induced gastritis were treated for 14 days with a combination regimen of acid inhibitory therapy and antibiotics. Latent, active and total MMP-9 levels decreased consistently and significantly by successful *H. pylori* eradication, in antrum as well as corpus mucosa, compared with those prior to treatment, irrespective of the therapy regimen used. When treatment failed, however, the elevated levels remained unchanged. MMP-2 levels did not show major alterations after *H. pylori* eradication therapy. In the antrum of gastritis patients, approximately three-fold higher MMP-9 levels were found compared with the corresponding corpus. As MMP-9 is predominantly secreted by inflammatory cells, this finding is consistent with the observation that the active inflammatory reaction, i.e. the number of infiltrating neutrophils and macrophages, in the antrum is similarly more intense compared with the corpus mucosa. The more severe antral inflammation is probably caused by a slow pyloro-cardial progression of gastritis because of a less dense *H. pylori* colonization of the corpus due to local acid production. *H. pylori*-

induced gastritis is accompanied by enhanced levels of mucosal cytokines, e.g. TNF-α and IL-8, that induce migration and activation of inflammatory cells and which are also capable of inducing the production of MMP-9 and, to a lesser extent, that of MMP-2 [8, 9]. Activation of the MMP-2 encoding gene by TNF-α and IL-8 is prevented, however, by the absence of an AP-1 binding site [2]. In summary, by successful *H. pylori* eradication, active and chronic inflammation decreased significantly in both antrum and corpus, accompanied by a considerable and significant decrease of latent, active and total MMP-9, particularly in the antrum.

The gelatinases MMP-2 and MMP-9 in gastric cancer

In **chapter 4 and 5**, two studies are presented regarding the presence of several MMPs and TIMPs in gastric cancer and their relationship with clinicopathological parameters, including survival. In the initial pioneer study, as reported in **chapter 4**, we assessed the levels of the gelatinases MMP-2 and MMP-9 in 50 gastric carcinomas and corresponding normal mucosa using quantitative gelatin zymography. In a majority of the gastric carcinomas the MMP-2 and MMP-9 levels were significantly enhanced compared with corresponding gastric mucosa, irrespective of the activity state of the enzymes. No relation was found with histopathological carcinoma classifications according to Laurén, the WHO and the TNM system. According to Cox's multivariate proportional hazards analyses, high MMP-2 and MMP-9 levels were of prognostic significance for a poor overall survival of the patients, independent of the major clinicopathological parameters.

MMPs and TIMPs in gastric cancer

In **chapter 5**, the results of a more comprehensive study are presented and discussed. The gelatinases MMP-2 and MMP-9 were assessed with new techniques in an expanded group of 81 gastric cancer patients, and MMP-7 and MMP-8 as well as TIMP-1 and TIMP-2 were included for comparison. Significantly enhanced levels of all MMPs measured and TIMP-1 were found in tumour tissue compared to normal gastric mucosa. Protein levels of MMP-7, MMP-8 and MMP-9 and the TIMPs showed some correlations with TNM stage, WHO and Laurén classification, but were not related with survival. An enhanced tumour MMP-2 level did not show a significant correlation with any of the clinicopathological parameters, but was confirmed to be an independent prognostic factor in gastric cancer. The consistent prognostic relevance of MMP-2 was underlined by the fact that both the old group of patients (n =50), described in

chapter 4, and the more recent group of patients (*n* =31) were independently subdivided based on a low or high MMP-2 antigen content of the carcinoma, using the same cut off value. Several immunohistochemical, zymographic and mRNA studies underscore the importance of MMP-2 as a prognostic indicator for gastric carcinoma patients [10-12]. In contrast to our initial study, high MMP-9 levels did not show a significant correlation with survival nor did the ratio MMP-9/TIMP-1, possibly related to the relatively small number of patients in the intial study and/or the relatively high MMP-9 levels in early gastric carcinomas.

NGAL in gastric cancer

Next to MMP-2 and MMP-9, the zymograms analysed revealed extra bands that are most likely heterodimers of MMP-9 with neutrophil gelatinase-associated lipocalin (NGAL). The results of an additional analysis to the presence of MMP-9/NGAL complexes in gastric cancer tissue and their possible clinical relevance are described in **chapter 6**. NGAL and MMP-9/NGAL complexes were determined in tissue homogenates from the same 81 gastric cancer patients analyzed in chapter 5 using specific ELISAs and bioactivity assays (BIA). NGAL and MMP-9/NGAL levels were significantly enhanced in gastric carcinomas compared to corresponding normal gastric mucosa. High levels of MMP-9/NGAL complexes in gastric tumours were significantly associated with worse survival in Cox's univariate and multivariate analysis, whereas the levels of NGAL and MMP-9 were not indicative for survival. Not just the enhanced presence, but more the activation state of the proteinase seems therefore crucial for prognosis. Immunohistochemically, MMP-9 as well as NGAL in gastric cancers were mainly present in either the neutrophils or the epithelial cells, depending on the individual cancer and on the location within the tumour. Immunofluorescence double-staining indicated that, although MMP-9 and NGAL were in general present in close proximity, overlap of MMP-9 and NGAL immunoreactivity, presumably indicating complex formation, was limited and mainly restricted to extracellular areas. The prognostic value of MMP-9/NGAL complexes is in accordance with the postulated role of NGAL in the protection of secreted MMP-9 against autolysis, hence contributing to an enhanced pool of potentially active MMP-9, a proteolytic enzyme associated with angiogenesis and tumour growth [5].

MMP and TIMP gene polymorphisms in gastric cancer

A study regarding the genotype distribution and allele frequencies of SNPs of MMP-2, MMP-7, MMP-8 and MMP-9 and TIMP-1 and TIMP-2 in 79 Caucasian gastric cancer patients in relation to tumour progression, patient survival and tissue antigen expression, is reported in **chapter 7**. The genotype distribution and allele frequencies were similar in gastric cancer patients and controls, except for MMP-$7_{-181A>G}$. In addition, the genotype distribution of MMP-$7_{-181A>G}$ was associated with *H. pylori* status and tumour-related survival of the patients. Single-nucleotide polymorphism TIMP-$2_{303C>T}$ correlated significantly with the WHO classification and also strongly with tumour-related survival. SNPs of MMP-2, MMP-8, MMP-9 and TIMP-1 were not associated with tumour-related survival. Only the gene promoter MMP-$2_{-1306C>T}$ polymorphism correlated significantly with the protein level within the tumours. First-order dendrogram cluster analysis combined with Cox analysis identified the MMP-$7_{-181A>G}$ and TIMP-$2_{303C>T}$ polymorphism combination to have a major impact on patients survival outcome.

Therapeutic MMP inhibition

Because overexpression of MMPs in different inflammatory and malignant gastrointestinal diseases facilitates angiogenesis and carcinogenesis, the correction of unbalanced MMP levels would be a straightforward target of treatment. A simple approach to achieve this would be the elimination or inhibition of a causative agent responsible for chronic inflammation and unbalanced expression of MMPs, for example *H. pylori* eradication in *H. pylori*-induced gastritis or altered intestinal flora in pouchitis. After successful *H. pylori* eradication indeed an improvement and normalization of the chronic inflammatory tissue response in the stomach was observed that was accompanied by a significant decrease of MMP-9 levels and almost unchanged MMP-2 levels. Many altered mucosal parameters that have been associated with gastric cancer and its prognosis, like growth factors and cytokines [13-15], plasminogen activators [16, 17] and superoxide dismutases [18, 19] show a reversal after successful *H. pylori* eradication. However, as only a minority of the patients with *H. pylori* gastritis develops gastric cancer on long-term, and since inflammation and genetic diversity might play an important role in cancer susceptibility [20, 21], the question remains whether eradication therapy will result in a decline of gastric cancer incidence. Another example is the decrease of enhanced MMP-1 and MMP-2 levels in patients with pouchitis that were treated with metronidazole [22].

If a causative agent cannot be removed, like in unsuccessful elimination of *H. pylori,* chronic inflammatory diseases and non-resectable cancer, inhibition of MMPs seems a

logical approach. Much effort therefore has been invested in search and development of synthetic MMP inhibitors. The currently known MMP inhibitors are divided into four classes: 1) Tissue Inhibitors of MetalloProteinases (TIMPs); 2) Tetracyclin-derivatives; 3) Peptide-based synthetic MMP inhibitors; and 4) Non-peptidic MMP inhibitors. Synthetic TIMPs are not suitable for oral administration due to their low molecular weight [23]. Tetracyclines have been shown to inhibit MMPs [24, 25] and especially minocycline appeared to be effective in rheumatoid arthritis [26, 27]. Peptidic MMP inhibitors, like batimastat and marimastat, have been developed that mimicked part of the peptide sequence surrounding the point in the collagen molecule first cleaved by interstitial collagenase allowing the inhibitor to fit tightly within the active site of the MMP. The zinc atom in this active site is subsequently chelated through a zinc-binding group [28]. A number of non-peptidic inhibitors like prinomastat has been developed with some efficacy in experimental cancer models [29]. The results of animal studies suggest a potential role for MMP inhibitors in chronic inflammatory diseases including pulmonary emphysema, multiple sclerosis, bacterial meningitis, graft-versus-host disease and colitis [30]. For example, in experimental models of colitis several MMP inhibitors induced decreased MMP-9 levels accompanied by decreased inflammatory scores [31-33]. Unfortunately, MMP inhibitors have not been proven successful in clinical trials for use in most chronic inflammatory diseases or cancer [34-40]. The only example of a clinically available MMP inhibitor for use in chronic inflammation is periostat, which is FDA-approved for use in periodontitis [41]. The only MMP inhibitor with some clinical efficacy in malignancy appeared to be marimastat, that showed a non-significant survival benefit in patients with non-resectable gastric cancer and a significant survival benefit in a sub-group of patients previously treated with chemotherapy (2-year survival of 5% in the placebo group and 18% in the treatment group, respectively)[40]. In higher dosages the use of marimastat was limited because of musculoskeletal side-effects like arthralgia, tendinitis and myalgia [42].

Perspectives

The studies in this thesis describe the clinical impact of several MMPs and TIMPs in *H. pylori*-induced gastritis and gastric cancer. MMP-2, MMP-7, MMP-8 and MMP-9, NGAL, MMP-9/NGAL and TIMP-1 were significantly increased in tumour tissue of gastric cancer patients compared to normal gastric mucosa whereas only enhanced levels of MMP-2 and MMP-9/NGAL complexes were independently related to worse prognosis. Several studies support the finding that MMP-2 is associated with tumour progression and prognosis in gastric cancer [10-12]. Overexpression of individual MMPs is frequently accompanied by a corresponding increased expression of TIMPs,

as has been shown for MMP-9 and TIMP-1 in lung cancer patients [43]. The question remains whether enhanced MMP and TIMP levels in gastric cancer result in more functional activity of the enzymes during cancer progression or are merely a sign of deregulated expression [for review see 5].

Preclinical studies have demonstrated that MMP-9 plays an important role in tumour-induced angiogenesis with tumour-associated inflammatory and stromal cells to be the main source of the proteinase. MMP-9-mediated release of vascular endothelial growth factor (VEGF) and recruitment of pericytes to the angiogenic vasculature have been postulated as major processes involved in host MMP-9 stimulated angiogenesis. Paradoxically, MMP-9 as well as other MMPs, including MMP-2, MMP-3, MMP-7 and MMP-13, are able to inhibit angiogenesis by proteolytic generation of endogenous inhibitors derived from extracellular matrix (ECM) proteins and non-matrix derived extracellular proteins. These endogenous inhibitors include tumstatin derived from the NC1 domain of type IV collagen, endostatin derived from type XVIII collagen and angiostatin generated from plasminogen. Both pro- and anti-angiogenic properties of MMP-7 have been reported in preclinical studies, as well as the ability to modify the function of proteins that are involved in tumour proliferation, apoptosis and invasion, such as epidermal growth factor (EGF) and tumour-necrosis factor-alpha (TNF-α) [5].

The lack of correlation between MMP-8 and outcome might be due to the anti-metastatic properties of MMP-8, as, for example, human breast carcinoma cells with metastatic potential had dramatically reduced expression of MMP-8 compared to non-metastatic cells [44].

The failure of broad-spectrum MMP inhibitors to improve survival in clinical cancer studies appears to be related to the more complex role of different MMPs in different stages of carcinogenesis than initially thought [45, 46] and has raised the necessity to select appropriate MMPs as drug targets [47]. Because of the enhanced expression in various human tumours, the association with invasiveness and the ability to degrade type IV collagen, MMP-2 is considered a potential target for inhibition. In experimental models it has been shown that MMP-2 is indeed associated with angiogenesis, tumour growth and metastasis [47]. Our results regarding the enhanced MMP-2 levels in gastric tumours, their independent correlation with prognosis and the correlation between the MMP-2$_{-1306C>T}$ polymorphism and tumour MMP-2 levels further support the view that MMP-2 is a potential drug target. Based on animal studies, MMP-7 has also been proposed as an anticancer drug target [47]. This is further substantiated by our finding of enhanced gastric tumour levels of MMP-7, the correlation with tumour stage and the correlation of the MMP-7$_{-181A>G}$ polymorphism with prognosis. MMPs -3 and -8 should not be inhibited because of their essential role in homeostasis and are therefore considered anti-targets. MMP-9 has pro-tumourigenic effects early in the malignant process stimulating angiogenesis but has anti-tumourigenic properties

in advanced disease, which makes it a difficult drug target. MMP-1, MMP-2, MMP-3, MMP-9, MMP-13 and MMP-14 are involved in cleavage and inactivation of CXCL12, a chemokine that attracts metastasizing cells, and inhibition of these MMPs might even stimulate metastasis [47, 48]. To understand the pleiotropic roles of MMPs in cancer *in vivo* and to select appropriate target MMPs, it is necessary to fully elucidate the MMP substrate degradome. This will facilitate the development of effective MMP inhibitors directed against target MMPs and avoiding anti-target MMPs [49]. An example of a selective MMP inhibitor is Ro-28-2653, an inhibitor with high selectivity for MMP-2, MMP-9 and membrane-type 1 (MT1)-MMP [50], that has recently been shown to decrease livermetastasis in an animal model of pancreatic cancer [51]. However, it remains a challenge to develop effective MMP inhibitors due to redundancy and similarities in MMP active sites. The clinical trials performed until now, have been carried out in advanced stages of aggressive cancers. A possible application is the use of MMP inhibitors earlier in the disease process in benign tumors and in secondary prevention of cancer, for example by chemoprevention of colorectal cancer in high-risk groups [52]. In experimental models, MMP inhibitors were effective in reducing metastatic cell growth and the metastasis-associated bone remodeling [53, 54]. One remaining therapeutic opportunity may therefore be the inhibition of matrix degradation in advanced cancers in order to limit bone metastases. Other techniques under investigation are RNA silencing technology for downregulation of endogenous MMP expression [55] and liposomal drug targeting against MT-MMP [56].

Conclusion

MMPs have important functions in normal physiology as well as in inflammatory processes and carcinogenesis, with distinct patterns of expression at different times and sites of progression. MMPs are associated with prognosis and are independent prognostic factors in gastrointestinal malignancies including gastric cancer. The concept that inhibition of matrix degradation could improve survival in gastrointestinal malignancy could not be substantiated until now, due to dose-limiting toxicity, the advanced stage of the cancer in the patients treated and the lack of evidence that inhibition of matrix degradation will result in inhibition of disease progression and improved survival. Assessment of MMP profiles at time of diagnosis by measurement of MMP protein levels and determination of selected SNPs of MMPs to select patients who would potentially benefit from (neo-)adjuvant therapy in gastrointestinal cancer has not been investigated until now, but deserves more attention [57]. The development of semi-selective MMP inhibitors aimed at target MMPs without musculoskeletal side effects in therapeutic dosages is another promising field of interest that is being

explored now. In addition, MMP inhibitors should be investigated for their use earlier in the malignant process and for use in combination with other therapeutic modalities [58].

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CHAPTER 9

Samenvattende discussie

Inleiding

Helicobacter pylori infectie van het maagslijmvlies veroorzaakt een chronische gastritis die de kans op benigne peptische ulcera en maagkanker vergroot. Ondanks de sterk gedaalde incidentie is maagkanker nog altijd de op één na meest voorkomende vorm van kanker wereldwijd, verantwoordelijk voor 10% van alle gevallen van kanker en 12% van de totale kankersterfte. Gemiddeld genomen is de vijfjaarsoverleving slechts 12% omdat de helft van de patiënten ten tijde van het stellen van de diagnose al metastasen heeft en er bij de andere helft slechts in 50% van de gevallen een in opzet curatieve resectie mogelijk is. Bij chronische ontstekingsprocessen en tumorgroei is er sprake van een ongecoördineerde afbraak en herstel van de extracellulaire matrix door proteasen, waaronder de matrix metalloproteïnasen (MMPs). In dit proefschrift staan enkele studies beschreven naar de klinische betekenis van de gelatinasen MMP-2 en MMP-9 bij chronische, door *H. pylori* veroorzaakte, gastritis alsmede het effect van eradicatietherapie hierop. Vervolgens worden onderzoeken besproken naar de klinische betekenis van MMPs, en hun natuurlijke inhibitoren TIMPs, bij maagkanker, alsmede de invloed van functionele genpolymorfismen van deze MMPs en TIMPs. Tenslotte worden de mogelijkheden en onmogelijkheden van remming van MMPs bij gastritis en maagkanker besproken.

Matrix metalloproteïnasen bij chronische gastritis en maagkanker

In een overzicht (**hoofdstuk 1**) staan de belangrijkste kenmerken en functies van MMPs, TIMPs en lipocalinen beschreven. In het kort samengevat, MMPs zijn proteolytische enzymen met een centraal zinkion, die in inactieve vorm uitgescheiden worden en geactiveerd dienen te worden door beperkte proteolyse voordat ze hun biologische functie kunnen uitoefenen. Ze worden geïnactiveerd door zogenaamde TIMPs: tissue inhibitors of metalloproteinases. TIMPs zijn natuurlijk voorkomende MMP remmers die complexen vormen met de meeste MMPs. Gebaseerd op overeenkomsten in eiwitstructuur en substraatvoorkeur worden de MMPs verdeeld in subgroepen, te weten collagenasen, stromelysinen, matrilysinen, gelatinasen, elastasen en membraan-type MMPs. De studies in dit proefschrift zijn gericht op MMP-2, MMP-7, MMP-8 en MMP-9 vanwege de veronderstelde klinische betekenis van deze MMPs bij gastritis en maagkanker. MMP-2 en MMP-9 zijn gelatinasen die specifiek basaalmembraan collageen type IV en gelatines afbreken. MMP-2 wordt vooral door stromacellen geproduceerd, terwijl MMP-9 met name uit ontstekingscellen zoals neutrofiele granulocyten vrijkomt. Neutrofiel collagenase (MMP-8) wordt gesynthetiseerd in neutrophiele granulocyten voor het verlaten van het beenmerg. Matrilysine (MMP-7) wordt vooral

afgescheiden door epitheliale- en tumorcellen. De lipocalinen bestaan uit een groep kleine eiwitten met uiteenlopende biologische functies waaronder regulatie van de celhomeostase en transport van eiwitten. Eén van deze lipocalinen is neutrofiel gelatinase-geassocieerd lipocaline (NGAL, lipocaline-2), dat wordt opgeslagen in secundaire granulae van neutrofiele granulocyten en dat heterodimeren kan vormen met neutrophil gelatinase-B (MMP-9).

De gelatinasen MMP-2 en MMP-9 bij door *Helicobacter pylori* geïnduceerde gastritis

Een studie naar de klinische betekenis van de gelatinasen MMP-2 en MMP-9 bij chronische, door *H. pylori* veroorzaakte, gastritis staat beschreven in **hoofdstuk 2**. Bij 45 patiënten met en 27 patiënten zonder *H. pylori* gastritis werden de MMP-2 en MMP-9 niveaus in maagbiopten gemeten. Bij patiënten met *H. pylori* gastritis werden zowel in antrum- als in corpusbiopten significant verhoogde MMP-9 niveaus gevonden ten opzichte van *H. pylori* negatieve controles. Deze verhoogde MMP-9 niveaus vertoonden een goede correlatie met de ernst van de ontsteking. Bij patiënten met een pangastritis werden in het corpus hogere MMP-9 niveaus gevonden dan bij patiënten met een antrale gastritis. Immunohistochemisch was MMP-9 voornamelijk gelocaliseerd in ontstekings- en stromacellen en in pariëtale cellen in het corpus. MMP-2 niveaus in maagbiopten van *H. pylori* positieve en negatieve patiënten verschilden nauwelijks van elkaar. Aangezien *H. pylori* gastritis geassocieerd is met het maagcarcinoom, en zowel bij *H. pylori* gastritis en het maagcarcinoom veranderde MMP-profielen worden gevonden, hebben wij een studie uitgevoerd naar het effect van *H. pylori* eradicatietherapie op deze MMP-profielen, zoals in **hoofdstuk 3** staat vermeld. Daartoe werden 58 patiënten met *H. pylori* gastritis behandeld met *H. pylori* eradicatietherapie, bestaande uit een combinatie van zuurremmende medicatie en antibiotica, gedurende 14 dagen. Na succesvolle eradicatietherapie was er sprake van een significante daling van MMP-9 niveaus in antrum- en corpusbiopten, terwijl na persisterende *H. pylori* infestatie deze nauwelijks veranderden. MMP-2 niveaus veranderden niet na succesvolle eradicatie.

De MMP-9 niveaus in het antrum bij patiënten met *H. pylori* gastritis liggen een factor drie hoger dan in het corpus. Aangezien MMP-9 voornamelijk gesecerneerd wordt door ontstekingscellen is dit verklaarbaar door de intensere ontstekingsreactie in het antrum vergeleken met die in het corpus. Dit hangt mogelijk samen met de langzame uitbreiding van antrum naar corpus van de gastritis als gevolg van een minder intense *H. pylori* infestatie van het corpus door locale zuurproduktie. Gastritis door *H. pylori* gaat gepaard met verhoging van mucosale cytokines die migratie en activatie van

ontstekingscellen veroorzaken en die tevens het vrijkomen van MMP-9, en in mindere mate van MMP-2, stimuleren. Activatie van het gen dat codeert voor MMP-2 door cytokines wordt namelijk verhinderd door de afwezigheid van een zogenaamde AP-1 bindingsplaats. Samenvattend neemt de actieve en chronische ontstekingscomponent in maagslijmvlies af na succesvolle *H. pylori* eradicatie, hetgeen gepaard gaat met een significante afname van MMP-9 niveaus, met name in het antrum.

De gelatinasen MMP-2 en MMP-9 bij maagkanker

De resultaten van een pionierstudie naar de klinische betekenis van de gelatinasen MMP-2 en MMP-9 bij het maagcarcinoom worden besproken in **hoofdstuk 4**. MMP-2 en MMP-9 niveaus werden gemeten in 50 maagcarcinomen en corresponderende normale maagmucosa door middel van gelatine-zymographie. Bij de meeste maagcarcinomen werd een verhoogd MMP-2 en MMP-9 niveau gemeten, zonder duidelijke relatie met type histologie of TNM-stadium. Als één van de eersten vonden we een verband tussen verhoogde MMP-2 en MMP-9 niveaus en slechtere overleving, onafhankelijk van de belangrijkste klinische- en pathologische variabelen.

MMPs en TIMPs bij maagkanker

De resultaten van een uitgebreidere vervolgstudie naar verschillende MMPs en TIMPs bij een grotere groep patiënten met een maagcarcinoom, onder gebruikmaking van modernere analysetechnieken, staan in **hoofdstuk 5** van dit proefschrift. Bij 81 patiënten werden naast MMP-2 en MMP-9 ook het MMP-7, MMP-8, TIMP-1 en TIMP-2 niveau gemeten. De niveaus van alle gemeten MMPs en van TIMP-1 waren in tumorweefsel significant verhoogd in vergelijking met normaal maagslijmvlies. Ongeacht de bepalingsmethode vonden we nog steeds een significant verband tussen hogere MMP-2 waarden in tumorweefsel en slechtere overleving, onafhankelijk van differentiatiegraad van de tumor en tumorstadium. Geen verband met de overleving werd gevonden voor de andere MMPs en voor de TIMPs, ook niet (meer) voor MMP-9.

De consistente prognostische betekenis van MMP-2 wordt onderstreept door het feit dat zowel de initieel onderzochte groep van 50 patiënten als de later toegevoegde groep van 31 patiënten onafhankelijk van elkaar onderverdeeld konden worden op basis van dezelfde discriminerende waarde van MMP-2 met betrekking tot de prognose. Inmiddels hebben verschillende immunohistochemische, zymographische en mRNA studies de klinische relevantie van MMP-2 als onafhankelijke prognostische factor bij het maagcarcinoom bevestigd. De relatie tussen een verhoogd MMP-9

niveau en overleving kon in deze uitgebreidere studie, met een grotere groep patiënten, niet bevestigd worden.

NGAL bij maagkanker

Naast de gelatinasen MMP-2 en MMP-9 bevatten de onderzochte zymogrammen van maagcarcinomen, extra banden veroorzaakt door heterodimeren van neutrofiel-gelatinase met lipocaline (NGAL of lipocaline-2). In **hoofdstuk 6** wordt een aanvullende analyse naar de presentatie van deze MMP-9/NGAL complexen in maagcarcinomen en de mogelijke klinische betekenis hiervan besproken. NGAL en MMP-9/NGAL complexen werden door middel van ELISAs en bioactiviteit-assays bepaald in weefselhomogenaten van dezelfde 81 patiënten als besproken in de studie van hoofdstuk 5. In maagcarcinomen werden significant verhoogde MMP-9, NGAL en MMP-9/NGAL niveaus gevonden vergeleken met normale maagmucosa. Hoge MMP-9/NGAL spiegels waren geassocieerd met slechtere overleving, terwijl NGAL en MMP-9 waarden niet geassocieerd waren met overleving. Deze bevindingen wijzen erop, dat niet zozeer de verhoogde presentatie van beide enzymen geassocieerd is met prognose, maar meer de mate van activiteit van MMP-9. MMP-9 en NGAL waren immunohistochemisch vooral gelocaliseerd in neutrofiele granulocyten en maagcarcinoomcellen. Immunofluorescentie dubbelkleuringen wezen echter uit dat, hoewel MMP-9 en NGAL in elkaars nabijheid voorkwamen, er slechts een beperkte co-localisatie van beide kleuringen was als maat voor complexvorming tussen beide factoren. De bevinding dat MMP-9/NGAL complexen geassocieerd zijn met overleving is in overeenstemming met de veronderstelde remming van de autolyse van MMP-9 door NGAL waardoor er een grotere hoeveelheid MMP-9 circuleert. MMP-9 stimuleert tumorangiogenese en tumorgroei.

MMP en TIMP genpolymorfismen bij maagkanker

Als laatste is er een studie gedaan naar het verband tussen genpolymorfismen in de MMP -2, -7, -8, -9 en TIMP -1, -2 genen en klinische- en histologische variabelen, zoals tumorstadium en overleving, bij patiënten met een maagcarcinoom. Genpolymorfismen zijn veranderingen in een stukje DNA, waarbij een enkel nucleotide in het genoom vervangen is door een ander. Er zijn aanwijzingen dat genpolymorfismen in MMP en TIMP genen de eiwitexpressie van MMPs en TIMPs kunnen beïnvloeden en vanuit Aziatische landen is beschreven dat zij de kans op het ontwikkelen van een maligniteit kunnen vergroten. Zoals in **hoofdstuk** 7 staat beschreven, vonden wij een

verschil in distributie van een genpolymorfisme in het MMP-7 gen (MMP-$7_{-181A>G}$) dat bovendien geassocieerd was met *H. pylori* status en overleving. Een genpolymorfisme in het TIMP-2 gen (TIMP-$2_{-303C>T}$) was naast overleving ook gecorreleerd met de differentiatiegraad van de maagcarcinomen. De aanwezigheid van beide genpolymorfismen was -onafhankelijk van klinische en histologische variabelen- geassocieerd met een slechtere overleving en alle patiënten met beide genpolymorfismen bleken binnen korte tijd te zijn overleden. Er bestond geen verband tussen genpolymorfismen van MMP-2, MMP-8, MMP-9 en TIMP-1 genen en overleving. Alleen het genpromoter MMP-$2_{-1306C>T}$ polymorfisme was significant gecorreleerd met het MMP-2 eiwitniveau in de tumor.

Therapeutische remming van MMPs

Omdat overexpressie van MMPs bij gastrointestale ontstekingsprocessen en maligniteiten de angiogenese en carcinogenese bevordert, lijkt correctie van deze afwijkende MMP expressie een aantrekkelijke behandelingsoptie. De meest eenvoudige oplossing bestaat uit het daar waar mogelijk elimineren van onderliggende oorzaken, zoals bijvoorbeeld *H. pylori* eradicatie bij *H. pylori* gastritis en correctie van veranderde darmflora door behandeling met metronidazol bij pouchitis. Na succesvolle *H. pylori* eradicatie trad er inderdaad een afname van de chronische ontstekingsreactie in het weefsel op, met een parallele daling van verhoogde MMP-9 niveaus. Een dergelijke normalisatie werd ook waargenomen bij afwijkende expressie van mucosale parameters die geassocieerd worden met maagkanker en prognose, zoals groeifactoren, cytokines, plasminogeenactivatoren en superoxide dismutasen. Aangezien slechts een minderheid van de patiënten met een door *H. pylori* veroorzaakte gastritis een maagcarcinoom ontwikkelt en genetische diversiteit de gevoeligheid voor het ontwikkelen van maagcarcinoom beinvloedt, blijft de vraag in hoeverre *H. pylori* eradicatie leidt tot een daling van de incidentie van het maagcarcinoom.

Veel onderzoek is verricht naar de ontwikkeling van MMP remmers. De MMP inhibitoren kunnen worden verdeeld in: 1. tissue inhibitors of metalloproteinases (TIMPs); 2. tetracyclinederivaten; 3. peptide-achtige MMP inhibitoren en 4. non-peptide-achtige MMP inhibitoren. Synthetische TIMPs zijn als gevolg van hun lage molecuulgewicht niet geschikt voor orale toediening. Tetracylines remmen MMPs en met name minocycline blijkt effectief bij reumatoide arthritis. De werkzaamheid van diverse MMP inhibitoren bij chronische ontstekingsziekten, zoals longemfyseem, multiple sclerosis, bacteriële meningitis, afstotingsreacties en colitis en bij kanker is gebleken uit dierexperimentele studies. Helaas bleken MMP inhibitoren in klinische studies niet werkzaam. De enige geregistreerde MMP inhibitor voor gebruik bij chronische ontste-

king is periostat voor gebruik bij periodontitis. De enige MMP inhibitor die in klinische studies bij maligniteiten enig effect ressorteerde, was marimastat. Bij patiënten met een inoperabel maagcarcinoom was er in de marimastat behandelde groep sprake van een niet-significante verbetering van de overleving. In een subgroep van eerder met chemotherapie behandelde patiënten was er wel sprake van een significante verbetering van de overleving (tweejaars overleving in de placebogroep 5% versus 18% in de behandelde groep). Het gebruik van marimastat werd echter beperkt door het optreden van spier- en gewrichtsklachten bij gebruik in hogere doseringen.

Toekomstige ontwikkelingen

De in dit proefschrift beschreven studies laten de klinische betekenis zien van verschillende MMPs, TIMPs en NGAL bij *H. pylori* gastritis en het maagcarcinoom. MMP-2, MMP-7, MMP-8, MMP-9, NGAL, MMP-9/NGAL complexen en TIMP-1 bleken significant verhoogd te zijn in tumorweefsel van patiënten met een maagcarcinoom in vergelijking met normale maagmucosa. Alleen verhoogde MMP-2 en MMP-9/NGAL niveaus bleken onafhankelijk van klinische en histologische variabelen geassocieerd met slechtere overleving. Ook uit andere studies blijkt de relatie van MMP-2 met prognose. Verhoogde expressie van individuele MMPs gaat vaak gepaard met een corresponderende verhoging van hun remmende TIMPs, zoals ondermeer beschreven is voor MMP-9 en TIMP-1 bij longkankerpatiënten. Het blijft de vraag of verhoogde MMP en TIMP expressie bij het maagcarcinoom resulteert in verhoogde activiteit van deze enzymen of anderszins slechts een uiting is van een verstoorde en gedereguleerde expressie. Uit preklinisch onderzoek is gebleken dat gelatinase-B (MMP-9) een belangrijke rol speelt bij tumorgeïnduceerde angiogenese en dat in de tumor gelocaliseerde ontstekings- en stromacellen de belangrijkste bron van MMP-9 zijn. Het onder invloed van MMP-9 vrijkomen van vasculaire endotheliale groeifactor (VEGF) wordt gezien als een van de belangrijkste processen bij door MMP-9 gestimuleerde angiogenese. Net als een aantal andere MMPs kan MMP-9 echter ook angiogenese remmen door proteolytische afsplitsing van endogene inhibitoren, waaronder tumstatine uit type IV collageen, endostatine uit type XVIII collageen en angiostatine uit plasminogeen. Het matrilysine MMP-7 heeft zowel pro- als anti-angiogene eigenschappen en kan de functie van eiwitten die betrokken zijn bij proliferatie, apoptose en invadering, zoals epidermale groeifactor (EGF) en tumor necrosis factor-α, beïnvloeden.

Het ontbreken van een correlatie tussen neutrofiel collagenase (MMP-8) en overleving kan verband houden met de anti-metastatische eigenschappen van MMP-8, omdat bijvoorbeeld de expressie van MMP-8 in een agressieve borstkankercellijn sterk verlaagd was in vergelijking met een minder agressieve cellijn.

Het gebrek aan effectiviteit in klinische studies van MMP inhibitoren lijkt samen te hangen met een meer complexe rol van de verschillende MMPs in de verschillende stadia van de carcinogenese dan aanvankelijk werd aangenomen en toont de noodzaak om MMPs te selecteren die in aanmerking komen voor selectieve remming. MMP-2 lijkt een geschikt doelwit voor remming vanwege de verhoogde expressie in tumoren, de correlatie met invasief gedrag van deze tumoren en het vermogen om gelatinase af te breken. In experimentele modellen blijkt MMP-2 inderdaad geassocieerd te zijn met angiogenese, tumorgroei en metastasering. De verhoogde MMP-2 niveaus in maagcarcinomen, de correlatie van het MMP-2$_{-1306C>T}$ genpolymorfisme met MMP-2 niveaus in de tumor en de onafhankelijke correlatie van MMP-2 niveaus in de tumor met prognose en overleving ondersteunen verder de gedachte dat remming van MMP-2 een geschikte interventie lijkt bij het maagcarcinoom.

Vanwege het verhoogde MMP-7 niveau in maagcarcinomen, de relatie met tumorstadium en de relatie van het MMP-7$_{-181A>G}$ genpolymorfisme met prognose, en op basis van gegevens uit dierexperimenteel onderzoek, lijkt ook MMP-7 een geschikt doelwit voor remming. MMP-3 en MMP-8 spelen een belangrijke rol in homeostase en dienen wellicht niet geremd te worden. MMP-9 stimuleert vroeg in het proces van maligne ontaarding de tumorgroei door toename van de angiogenese maar heeft later in het ziekteproces tumorremmende eigenschappen door vorming van angiostatine, tumstatine en endostatine. MMP-1, MMP-2, MMP-3, MMP-9, MMP-13 en MMP-14 zijn betrokken bij de splitsing en inactivatie van CXCL12, een chemokine dat metastaserende kankercellen aantrekt. Remming van deze MMPs zou zelfs metastasering kunnen bevorderen. Om de onderscheiden functies van MMPs te kunnen begrijpen, teneinde MMPs te kunnen selecteren die in aanmerking komen voor selectieve remming, is het belangrijk het volledige substraat van MMPs bij maligniteiten *in vivo* in kaart te brengen. Een voorbeeld van een veelbelovende selectieve MMP inhibitor is Rp-28-2653, die selectief MMP-2, MMP-9 en membraan-type 1 (MT1)-MMP remt en levermetastasen remt in een diermodel voor het pancreascarcinoom. De ontwikkeling van MMP inhibitoren blijft echter een uitdaging vanwege de overlap in actieve plaatsen van MMPs. De weinig succesvolle klinische studies die tot dusverre met MMP inhibitoren uitgevoerd zijn vonden alle plaats bij patiënten met voortgeschreden of uitbehandelde maligniteiten. Omdat MMP inhibitoren ingrijpen in de tumorangiogenese, verdient het overweging MMP inhibitoren vooral in te zetten in een vroeg stadium van de ziekte, bij premaligne aandoeningen (bijvoorbeeld bij Familiaire Adenomatosis Coli patiënten met dunne-darmadenomen), en als secundaire preventie van kanker bij risicogroepen. Aangezien uit experimenteel onderzoek gebleken is dat MMP inhibitoren de groei van metastaserende cellen en de metastase gerelateerde botombouw remden, is een andere mogelijke toepassing het gebruik bij patiënten met botmetastasen ter vertraging van de botafbraak. Andere technieken die onder-

zocht worden zijn RNA silencing technologie ter downregulatie van endogene MMP expressie en liposomale drug targeting tegen MT-MMP.

Conclusies

MMPs hebben belangrijke functies bij fysiologische- en pathologische processen zoals chronische ontstekingen en maligniteiten. Het expressiepatroon van MMPs varieert tussen verschillende tumortypen en stadia in het ziekteproces en meerdere MMPs, onder meer MMP-2 bij het maagcarcinoom, zijn onafhankelijk van klinische of histologische factoren geassocieerd met prognose. Het concept van MMP inhibitie als werkzame behandeling bij maligniteiten kon tot op heden nog niet vastgesteld worden als gevolg van dosisremmende toxiciteit, het onderzoeken bij een te ver voortgeschreden ziekteproces, en een gebrek aan bewijs dat brede MMP inhibitie resulteert in vertraging van ziekteprogressie en verbetering van de overleving.

Het verdient aanbeveling om te onderzoeken of bij het maagcarcinoom door middel van een bepaling van het MMP-profiel ten tijde van het stellen van de diagnose (bijvoorbeeld door het bepalen van eiwitniveaus in biopten uit de tumor en het bepalen van relevante genpolymorfismen in DNA uit perifere leucocyten) subgroepen van patiënten geïdentificeerd kunnen worden met een slechte prognose. Vervolgens dient dan beoordeeld te worden welke subgroepen van patiënten het meest profijt hebben van neo-adjuvante chemotherapie en adjuvante chemoradiatietherapie. Bij klinisch onderzoek naar de effectiviteit van de semi-selectieve MMP inhibitoren die op dit moment ontwikkeld worden ligt het voor de hand te stratificeren voor deze subgroepen.

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Nawoord

"Het is pas klaar als het af is."

Het was vaak bijna klaar maar nog niet af. Daarmee heeft het schrijven van een proefschrift veel weg van het produceren van een speelfilm. Een belangrijk verschil tussen een speelfilm en een Leids proefschrift is dat in de aftiteling van een Leids proefschrift de naam van de regisseur ontbreekt. Desondanks zal blijken uit de aftiteling van deze speelfilm dat velen voor en achter de camera een steentje eraan hebben bijgedragen.

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Sjam Ganesh en Raymond Smeets staan vandaag naast mij als paranimfen.

Beste Sjam, jouw promotie-onderzoek vormde de basis voor enkele studies in dit proefschrift. Net als in 1994 zijn we weer elkaars buurmannen in het ziekenhuis en nu ook maten, en ik vind dat heel bijzonder.

Beste Raymond, in 1996 had ik de eer bij jouw promotie paranimf te mogen zijn. Elf jaar later zijn de rollen omgedraaid en staan we eindelijk quitte.

Beste pap en mam, met vooruitziende blik hebben jullie in 1968, het jaar dat ik begon in de eerste klas van de Mariaschool, een baksteentje bijgedragen aan een handtekeningenactie voor de vestiging van een medische faculteit in Maastricht. Soms verbaasd, maar altijd enthousiast, hebben jullie mijn keuzes ondersteund en daar ben ik jullie nog steeds heel dankbaar voor.

Lieve Thijs, bovenop de Etna heb jij de prachtige foto van gloeiende lava gemaakt, die symbool staat voor het onderwerp van dit proefschrift. Vlak nadat je de foto gemaakt had spetterde de lava naast ons neer. Je hebt daarmee niet het moeilijkste, maar wel het gevaarlijkste deel van het proefschrift voor je rekening genomen.

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boekje!" was je devies en daar heb je me op alle fronten bij geholpen. Ik dank je voor je grote geduld en steun en het is ook door jou dat ik hier nu sta. Het is af.

Curriculum vitae

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1962	geboren te Geleen
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1980-1981	Scheikundige Technologie, Technische Hogeschool, Eindhoven
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1982-1985	Student-assistent, Capaciteitsgroep Pathologie (Prof.dr. F.T. Bosman), project "Peptide hormone production in APUD-omas and in carcinomas containing neuroendocrine cells"
1984	Wetenschapsstage Institut für Pathologie, Universität Basel (Prof. dr.med. Ph.U. Heitz), onderwerp: "Immunohistochemical localization of substance P and serotonin in the human gastrointestinal tract"
1984-1987	Redactielid Tijdschrift voor openbare orde, rampenbestrijding en civiele verdediging "Alert", Staf voor de Civiele Verdediging, Ministerie van Binnenlandse Zaken, Den Haag
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