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Matrix metalloproteinases in gastric inflammation and cancer : clinical relevance and prognostic impact

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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 initial 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 liver metastasis 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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