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CHAPTER 2

MMP-2, MMP-8, MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL) in the colorectal cancer sequence

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ABSTRACT

Background and aim of the study: Matrix metalloproteinases (MMPs) have been implicated in colorectal cancer progression and prognosis. However, the role of MMPs in early cancer development and in the process of dissemination of the disease has been studied less extensively. In the present study, we investigated the expression and activity of MMP-2, -8 and -9 and neutrophil gelatinase-associated lipocalin (NGAL) in tissue from various stages of colorectal cancer progression.

Methods: The expression of NGAL, MMP-8, -2 and -9 was measured by ELISA in a series of normal colorectal mucosa, adenomatous polyps, adenocarcinomas, liver metastasis and normal liver (n= 20 of each group), and the activity of MMP-9 was measured by Bio–Immuno-Activity Assay (BIA) and quantitative gelatin-zymography of tissue homogenates. The degree of inflammation was assessed by myeloperoxidase (MPO) activity.

Results: There was a consistent and significant step-wise increase in the MMP-8 level associated with the progression from normal mucosa to adenoma (2-fold) and from adenoma to carcinoma (8-fold), with similar high levels in the liver metastases as in the primary carcinomas. We found a similar, though less pronounced, increase (up to 3-fold) in MMP-9 in this malignancy sequence, with a good correlation between the ELISA and zymography results (r = 0.60). NGAL also showed an increase in the normal mucosa - adenoma (4-fold) - carcinoma (6-fold) - liver metastasis (3-fold) sequence. The MMP-2 level only showed an increase (up to 1.5-fold) in the carcinomas. Interestingly, the primary colorectal carcinomas contained a significantly (P < 0.05) higher percentage of both MMP-2 and MMP-9 in the active enzyme form than adenomas and liver metastases (respectively 55 ± 6 vs 29 ± 3 and 30 ± 3, and 21 ± 1 vs 14 ± 2 and 10 ± 3). Normal liver tissues had low MMP levels.

Conclusion: In conclusion, the development of colorectal cancer, as illustrated by the normal mucosa - adenoma - carcinoma - liver metastasis sequence, is accompanied by a concurrent increase in the NGAL and MMP levels, particularly MMP-8 and MMP-9, providing evidence that they are causatively involved in colorectal cancer progression.
INTRODUCTION

Colorectal cancer is the third most frequent cancer worldwide, with more than 1.2 million new cases each year and 608,000 deaths in 2008 (source: World Health Organization, www.WHO.int). Up to 15% of these cancers are clustered within families, while the other 85% are classified as sporadic colorectal cancer. In both familial and sporadic cases, the majority of these cancers develop from a precursor lesion, the adenomatous polyp, in which sequential changes eventually lead to the development of cancer\(^1\),\(^2\).

Matrix metalloproteinases (MMPs) are metal-dependent proteolytic enzymes that play an important role in various biological processes as they determine the rate of remodelling of the extracellular matrix and are implicated in the regulation of angiogenesis, migration, invasion and cancer-cell growth and cell death\(^3\),\(^4\). Approximately 25 members of the MMP family have so far been identified, which have been numbered in the order of their discovery and subdivided based on the characteristics of their substrate specificity in collagensases, gelatinases, stromelysins, matrilysins and membrane-type MMPs\(^5\). Altogether, the members of the MMP family are able to degrade virtually all components of the extracellular matrix, such as collagens, laminin, fibronectin, vitronectin, entactin and proteoglycans\(^5\). Most MMPs are up-regulated in malignancy and, in most cases, are thought to contribute to cancer progression and dissemination. However, this is not always the case, as illustrated by the protective effects of overexpression of some MMPs (e.g., MMP-8 or neutrophil collagenase) that has been described for several tumours\(^6\),\(^7\),\(^8\).

Lipocalins, together with the fatty-acid-binding proteins (FABPs) and avidins, belong to the calycins\(^9\). These lipocalins are involved in the regulation of cell homeostasis, modulation of the immune response, and clearance of endogenous and exogenous compounds\(^9\). Neutrophil gelatinase-associated lipocalin (NGAL) is a 25 kD protein of human neutrophils, that is in part covalently complexed with MMP-9 (gelatinase B, 92 kDa type IV collagenase). It is located in specific granules apart from gelatinase granules in human neutrophils\(^10\).

In colorectal carcinomas, increased levels of active MMP-2 (gelatinase A, 72 kDa type IV collagenase) and MMP-9 have been reported compared to normal mucosa\(^11\)-\(^13\). In addition, a role in early carcinogenesis has been suggested for MMP-9, but not for MMP-2\(^14\)-\(^16\).

The aim of the present study was to investigate the presence and activity of MMP-2, MMP-8, MMP-9 and NGAL throughout the sequence from normal tissue, via adenomas and colorectal cancer, to liver metastases from colorectal origin. We used several techniques, including bio-immuno-activity assay (BIA), ELISA and quantitative zymography, to quantify the total and active amount of the proteinases.
PATIENTS, MATERIALS AND METHODS

Patients and Tissues

Adenomas (n=20) were freshly obtained by endoscopic snare polypectomy at the Department of Gastroenterology and Hepatology of the Leiden University Medical Center (LUMC). Fresh tissue samples of the adenomas were frozen and stored at -70°C until extraction. Part was routinely formalin fixed and embedded in paraffin for histological evaluation. Fourteen adenomas were found to be tubulovillous, five were tubular and one was villous. The degree of epithelial cell dysplasia was low grade in 14 adenomas and high grade in 6 adenomas.

In addition, fresh tissue was obtained from 20 patients who underwent resection for primary colorectal carcinoma at the Department of Surgery of the Leiden University Medical Center as previously described 17. 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 and parallel samples were routinely fixed and embedded in paraffin. Using the Dukes’ classification as modified by Astler and Coller 18, the carcinomas were classified in stage B1 (n=4), B2 (n=8), C1 (n=2), C2 (n=3) and D (n=3). Differentiation grade was poor in 2, moderate in 13 and well in 5 carcinomas. Four out of the 20 carcinomas were of the mucinous type.

Finally, fresh tissue was collected from 20 patients who underwent partial liver resection because of colorectal liver metastasis as previously described19. Representative samples of the carcinomas and of macroscopically normal liver tissue were frozen and stored at -70°C, other parts were routinely formalin fixed and embedded in paraffin. In all cases, the diagnosis metastasis of an adenocarcinoma from colorectal origin was confirmed by histopathological examination.

Tissue extraction and protein concentration

Tissue specimens were homogenized in 0.1 M Tris-HCl, 0.1% (v/v) Tween 80 buffer (pH 7.5), as extensively described previously17, 20, 21. Briefly, the samples were homogenized in 1 ml buffer per 60 mg wet tissue at 0°C. The homogenates were centrifuged at 8000 g for 2.5 min at 4°C and the supernatant was stored at -70°C until analysis. The protein concentration of the supernatant was determined by the method of Lowry22.

MMP-2 and MMP-9 gelatin zymography

Presence of active and latent forms of matrix metalloproteinases was analyzed by zymography on 10% SDS-polyacrylamide gels containing 2% gelatin and overnight incubation at 37°C, as described previously (12, 24). Sample volumes were adjusted to obtain a uniform protein content of 10 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 a LKB Ultroscan XL enhanced laser densitometer (633 nm). Two amounts (12 and 24 µg protein of an internal standard preparation, i.e. an 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).

NGAL, MMP-2, MMP-8 and MMP-9 ELISAs
The total amount of NGAL, MMP-2, MMP-8 and MMP-9 was determined, in appropriate dilutions of the homogenates, by established sandwich-ELISAs, as described previously. Results were expressed in ng MMP per mg protein.

MMP-2 and MMP-9 Bio-Immuno-Activity Assay (BIA)
Total MMP-2 and MMP-9 was also measured using a colorimetric assay. Antibodies to MMP-2 and MMP-9, as in the ELISAs, were used as catching antibody for the respective MMPs in the homogenates, during overnight incubation. These MMPs were then activated by incubation with 0.5 mM p-aminophenyl-mercuric acetate at 37°C and their activity measured with modified pro-urokinase (Ukcol) and S-2444 (0.6 mM), as chromogenic substrate, in assay buffer at 37°C. Reactions were performed in 96-well flat-bottomed microtitre plates, and a Titertek Multiskan photometer was used to follow the absorbance change at 405 nm. Results were calculated from a standard curve and expressed as units MMP per mg protein.

Myeloperoxidase activity
Myeloperoxidase (MPO) in tissue homogenates was assessed according to the procedure described by Kruidenier et al. In short, 50 µl tissue homogenate was incubated in buffer with 0.026% ortho-dianisidine dihydrochloride and 0.02% hydrogen peroxide. The reaction kinetics were followed in colorimetrically at 450 nm in microtitre plates. MPO was expressed in Arbitrary Units (AU) per mg protein.

Statistical analysis
Group means are given as mean ± standard error of the mean (SEM). Differences between dependent samples from 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. For testing the significance of a difference in means for independent samples the independent-samples Student’s t-test was used. Differences were considered significant when $P < 0.05$ (SPSS for Windows Release 7.0, 1995, SPSS Inc., Chicago, U.S.A.).
RESULTS

Myeloperoxidase activity

Inflammation, as assessed by the myeloperoxidase activity, was significantly higher \((P \leq 0.001)\) in adenomas \((4.72 \pm 0.35)\) compared to normal mucosa \((1.99 \pm 0.28)\), colorectal cancer \((2.14 \pm 0.30)\), liver metastases \((1.92 \pm 0.37)\) and normal liver tissue \((0.51 \pm 0.12)\). No relevant difference was observed between carcinomas and metastases.

MMP-8

There was a consistent and significant step-wise increase in the level of MMP-8 in the normal mucosa-adenoma (2-fold)-carcinoma (8-fold) sequence, with a similar level in the liver metastases compared to the primary carcinomas (Figure 1). Even after correction for inflammation by the MPO content, the increase of MMP-8 in carcinoma and liver metastasis persisted (Figure 2).

Figure 1. MMP-8 and NGAL, as measured by ELISA, in the colorectal cancer sequence. * \(P \leq 0.05\) vs. normal mucosa; ** \(P \leq 0.01\) vs. normal mucosa; ## \(P \leq 0.01\) vs. adenoma.
Inflammation, as assessed by the myeloperoxidase activity, was significantly higher ($P \leq 0.001$) in adenomas (4.72 ± 0.35) compared to normal mucosa (1.99 ± 0.28), colorectal cancer (2.14 ± 0.30), liver metastases (1.92 ± 0.37) and normal liver tissue (0.51 ± 0.12). No relevant difference was observed between carcinomas and metastases.

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Neutrophil granulocyte-associated lipocalin (NGAL)

Adenomas (4-fold) and carcinomas (6-fold) showed a marked increase in NGAL content compared to normal mucosa and resembled the trend of MMP-9 (Figure 1). Liver metastases were found to contain more NGAL than normal liver. After correction for inflammation the primary colorectal carcinomas still contained 4 times - and liver metastases 2 times - the amount of NGAL present in normal colonic mucosa (Figure 2).

MMP-9

The MMP-9 ELISA showed a comparable, though less pronounced, increase (up to 3-fold) in the sequence normal mucosa-adenoma-carcinoma (Figure 3). The MMP-9 content in liver metastases was lower than in the primary tumours and comparable to that in adenomas. After correction for inflammation, the colorectal carcinomas still contained consistently higher amounts of MMP-9 (Figure 2). Quantitative zymography showed significantly higher amounts of pro-, active- and total MMP-9 in carcinomas compared to the other tissues. The percentage of active MMP-9 in the carcinomas (21 ± 1%) was considerably higher compared to adenomas and liver metastases (14 ± 2% and 10 ± 3%, respectively), but lower than in normal colon mucosa (30 ± 4%).

Figure 2. Levels of MMP-8, NGAL (left axis), and MMP-9 (right axis), as determined by ELISA, in colorectal neoplasia, corrected for MPO activity. Values are expressed in ng NGAL or MMP/MPO. * $P \leq 0.05$ vs. normal mucosa; ** $P \leq 0.01$ vs. normal mucosa; # $P \leq 0.05$ vs. adenoma; ## $P \leq 0.01$ vs. adenoma.

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Figure 3. MMP-9 in the colorectal cancer sequence. Left axis: quantitative gelatin zymography (Z), results expressed in activity units (AU) and ELISA, results expressed in ng per mg protein. Right axis: BIA, results expressed in U/mg protein. * $P \leq 0.05$ vs. normal mucosa; • $P \leq 0.05$ vs. other tissues; •• $P \leq 0.01$ vs. other tissues; † $P \leq 0.05$ vs. normal liver. The percentages indicate the percentage active MMP-9 of the total amount of MMP-9 in the zymographic analyses.

BIA results were comparable to ELISA and quantitative zymography data (Figure 3). The highest MMP-9 content was found in the primary colorectal carcinomas. Liver metastases showed a lower MMP-9 content, comparable to adenomas. The overall correlation between ELISA, quantitative zymography and BIA was good, ranging from 0.60 ($P < 0.001$) between ELISA and zymography to 0.69 ($P < 0.001$) between BIA and zymography.

Figure 4. MMP-2 in the colorectal cancer sequence. Left axis: quantitative gelatin zymography (Z), results expressed in activity units (AU). Right axis: ELISA and BIA, results expressed in ng per mg protein (ELISA) and x10$^{-1}$ U/mg protein (BIA). * $P \leq 0.05$ vs. normal mucosa (total enzyme); ** $P \leq 0.01$ vs. normal mucosa (active enzyme); ## $P \leq 0.01$ vs. adenoma (active enzyme); †† $P \leq 0.01$ vs. normal liver (active enzyme); ••• $P \leq 0.001$ vs. other tissues. The percentages indicate the percentage active MMP-2 of the total amount of MMP-2 in the zymographic analyses.
MMP-2

The MMP-2 levels showed only an increase (up to 1.5-fold) in the carcinomas compared to normal mucosa (Figure 4). The MMP-2 level in liver metastases showed a comparable increase to the carcinomas when assessed by zymography, but not by the other techniques. The percentage of active enzyme in carcinomas (55 ± 6%) was significantly higher than in adenomas (29 ± 3%) and liver metastases (30 ± 3%). Normal liver tissue homogenates were found to contain lower levels of MMP-2 than any of the other tissues, although this difference was significant only in the ELISA results. The correlation between ELISA and gelatin zymography for MMP-2 was good ($r = 0.57; P < 0.001$), between BIA and ELISA the correlation was moderate ($r = 0.32, P < 0.005$), and relatively weak between the BIA and quantitative gelatin zymography ($r = 0.24, P < 0.05$).

![MMP-2](image)

Figure 4. MMP-2 in the colorectal cancer sequence. Left axis: quantitative gelatin zymography (Z), results expressed in activity units (AU). Right axis: ELISA and BIA, results expressed in ng per mg protein (ELISA) and $x10^{-1}$ U/mg protein (BIA). * $P \leq 0.05$ vs. normal mucosa (total enzyme); ** $P \leq 0.01$ vs. normal mucosa (active enzyme); ### $P \leq 0.01$ vs. adenoma (active enzyme); †† $P \leq 0.01$ vs. normal liver (active enzyme); *** $P \leq 0.001$ vs. other tissues. The percentages indicate the percentage active MMP-2 of the total amount of MMP-2 in the zymographic analyses.
DISCUSSION

Several *in vitro* and *in vivo* studies, reviewed by Mook et al.\textsuperscript{28}, have shown the relevance of MMP-2 and MMP-9 in colorectal cancer progression and metastasis. Both gelatinases are in principle secreted as inactive pro-enzymes. Activation of these pro-MMPs takes place through interaction with other MMPs and/or other proteases\textsuperscript{28}. Quantitative zymography has been shown previously to be an extremely reliable and sensitive technique for the detection of these gelatinases and to distinguish between the proenzyme and active form\textsuperscript{29, 30}. Using this technique, we found that pro-, active- and total MMP-2 and -9 levels are higher in carcinomas compared to normal colonic mucosa. These findings are in agreement with several other reports\textsuperscript{12, 16, 31-35}.

In the present study, we have also looked into the expression of MMPs in the early and later stages of colorectal carcinogenesis. We found that tissue levels of MMP-2, -8, and -9 and NGAL are increased in carcinomas and liver metastases of colorectal carcinomas, even after correction for inflammation, and that MMP-8, MMP-9 and NGAL are increased in colorectal adenomas as well.

Several studies already suggested a role for MMP-9 in early carcinogenesis, whereas MMP-2 seems to be of importance later in the adenoma-carcinoma sequence, once a carcinoma has developed. An up-regulation of MMP-9 in colorectal adenomas compared to the normal mucosa was found in two other zymography studies\textsuperscript{14, 16}. Up-regulation of the latent or active form of MMP-2 has never been observed in precancerous lesions. A trend towards a step-wise increase of MMP-9 in the colorectal adenoma-carcinoma sequence was also demonstrated in immunohistochemical studies of adenoma and carcinoma tissue\textsuperscript{15, 36} and these results were confirmed by performing real time RT-PCR on the same tissue specimens\textsuperscript{15}. MMP-9 expression in adenomas appears to be correlated with severity of dysplasia, but not with histological subtype\textsuperscript{14, 16, 37, 38}. In accordance with the abovementioned studies, we found a significant increase of total and active MMP-9 in colorectal carcinoma versus normal tissue measured either by ELISA, BIA or zymography. A trend towards increased expression of MMP-9 was observed in adenomas compared to normal mucosa, but this did not reach statistical significance. MMP-2 was only moderately up-regulated in colorectal cancer but not adenomatous tissue.

The formation of adenomas was further studied in APC-Min mice\textsuperscript{39} after knocking out either the MMP-2 or the MMP-9 gene. Ablation of the MMPs did not influence adenoma size; however, adenoma number was reduced by 40% and proliferation was reduced by 50% in the MMP-9\textsuperscript{-/-} mice compared to their MMP-9\textsuperscript{+/+} littermates\textsuperscript{40}. Knockout of MMP-2 did not reduce adenoma number or the number of proliferating cells. These findings support the hypothesis that there is a role for MMP-9, but not for MMP-2, early in the adenoma-carcinoma sequence.
MMP-8 (neutrophil collagenase, collagenase-2) is a 75 kDa neutrophil-derived matrix metalloproteinase that degrades type I, II en III collagen and a wide range of non-collagenous substrates like serine protease inhibitors and chemokines. Several studies in breast cancer, lung cancer and melanoma have shown that increased expression of MMP-8 can play a protective role in the progression of metastasis of cancer, probably through regulation of the inflammatory process induced by carcinogens and the modulation of tumour cell adhesion and invasion. These findings illustrate that the influence of MMP-8 on cancer varies between cancer types and throughout the cancer process, which makes it unsuitable as a target for anticancer drugs. Little is known about its role in colorectal cancer. Our results show that MMP-8 is significantly increased in adenomas, carcinomas and liver metastases compared to normal mucosa. The consequence of MMP-8 overexpression in the colorectal cancer sequence, i.e., protection or contribution to carcinogenesis, needs to be further explored.

NGAL (or neutrophil gelatinase-associated lipocalin, also called lipocalin-2 or human neutrophil lipocalin [HNL]) is a 25 kDa glycosylated protein that forms homo-dimers in neutrophil granules and heterodimers with MMP-9. It prevents inactivation of MMP-9 by binding to the gelatinase and thereby preventing its degradation, is a carrier of small lipophilic ligands, e.g. N-formyl peptides, and is involved in migration processes. NGAL was significantly enhanced in adenomas and carcinomas compared to normal mucosa. In liver metastases, NGAL levels were lower than in the carcinomas.

Neutrophil granulocytes are a major source of NGAL, MMP-8 and MMP-9 secretion. After correction for inflammation, the levels of both MMPs and NGAL were not significantly different in normal colon tissue and adenomas, suggesting that the increased expression in adenomas can mainly be attributed to an increased neutrophil influx. In carcinomas and liver metastases, however, the increase in MMP-8, MMP-9 and NGAL levels persisted even after correction for inflammation. These findings demonstrate that an influx of neutrophils into the tumour microenvironment is only partially responsible for the increase in MMP-8 and MMP-9 in the tumour and indicate that there is a true increase in the production of these proteins within the malignant tissues. In a series of gastric cancer patients, MMP-9 and NGAL-staining was present in a substantial part of epithelial cells. Also in colorectal cancer specimens, intensive epithelial NGAL staining was seen in the transitional mucosa between normal and malignant tissue, whereas occasionally MMP-9 positivity was observed in endothelial cells and incidentally in muscle cells, macrophages and fibroblasts. Several studies suggest that NGAL protects MMP-9 from auto-degradation by the formation of a NGAL-MMP-9 complex and thereby increases MMP-9 activity. With and without correction for inflammation, we found that NGAL showed -not surprisingly- the same pattern in tissue values as MMP-9.

Pyke et al. demonstrated the presence of mRNA of MMP-2 in fibroblasts/fibroblast-like cells and of MMP-9 mRNA in macrophages in cancer cell surrounding stromal tissue, but
not in the cancer cells themselves. In an experimental mouse model, using the short hairpin RNA interference technology (shRNA), Gerg et al.\(^4\) were able to demonstrate that MMP-2 and -9 are also expressed by colorectal cancer cells themselves. Furthermore, distinct roles were identified for the two gelatinases in the development of colorectal cancer metastases; MMP-9 is important in the process of extravasation and invasion, whereas MMP-2 influences the outgrowth of metastases but not their formation.

The liver is the main site of metastasis formation in colorectal cancer. The presence of MMP-2 and MMP-9 in colorectal liver metastasis has already been demonstrated by immunohistochemistry, in situ hybridization and zymography\(^49\text{-}^54\). In almost all cases, expression of MMP-2 and MMP-9 in metastases was higher than in normal liver tissue. We found that the MMP-9 and NGAL content of liver metastases from colorectal origin are lower than in the primary tumour, whereas MMP-2 and MMP-8 were expressed at an equal level. The percentage of active MMP-2 and MMP-9 was found to be lower in liver metastases than in the primary colorectal carcinoma and equalled the activity percentage found in adenomas. We speculate that the higher percentage of active enzyme reflects an increased proteolytic activity in the primary colorectal cancer, contributing to their invasive capacity. Few studies have compared the MMP-2 and -9 expression in liver metastases and in the primary tumour. In partial concordance with our results, Gentner et al.\(^5\) found lower mRNA expression of all measured MMPs (including MMP-2, -8 and -9) in liver metastases compared to the primary colorectal cancers derived from the same patients. These findings were not confirmed by Asano et al.\(^5\) who also used RT-PCR to quantify the mRNA expression of several MMPs in liver metastases; and although MMP-1, -10 and -11 expression was found to be decreased in the liver metastases compared with primary colorectal cancers, no difference in the expression of MMP-2, MMP-8 and MMP-9 was found. A dot blot hybridization study also showed no difference between mRNA levels of MMP-2 and MMP-9 in colorectal cancer or liver metastases\(^5\). Gelatinase-activity was increased in colorectal cancer in comparison to normal colorectal tissue, but the activity was lower in hepatic metastases\(^5\). It is therefore possible that up-regulation of these MMP-proteins occurs at a postranscriptional level.

In conclusion, our results show that the development of colorectal cancer, as illustrated by the normal mucosa – adenoma – carcinoma – liver metastasis sequence, is accompanied by a concurrent increase in the MMP levels which might contribute to the invasive process. Our findings suggest different roles for MMP-2, -8 and -9 throughout this process, which may have implications for the use of anti-MMP therapies in different stages of colorectal cancer.

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REFERENCES


