Tissue levels of active matrix metalloproteinase-2 and -9 in colorectal cancer

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The bioactivity of matrix metalloproteinases was studied in tissues from colorectal cancer patients by means of both quantitative gelatin zymography and a fluorometric activity assay. Next to paired samples of tumour tissue and distant normal mucosa (n=73), transitional tissue was analysed from a limited (n=33) number of patients. Broad-spectrum matrix metalloproteinase activity and both the active and latent forms of the gelatinases matrix metalloproteinase-2 and -9 were higher in tumour than in normal mucosa. The ratio’s between active and latent forms of matrix metalloproteinase-2 and -9 were highest in tumour tissue and normal mucosa, respectively. Matrix metalloproteinase-2 levels, both active and latent forms, correlated inversely with stage of disease, the tumours without synchronous distant metastases containing significantly (P=0.005) more active matrix metalloproteinase-2 than the others. At much lower levels of activity, the same trend was observed in distant normal mucosa. The level of latent form of matrix metalloproteinase-9 in tumour depended on tumour location. Neither the active form of matrix metalloproteinase-9 nor broad-spectrum matrix metalloproteinase activity in tumour tissue did correlate with any of the clinicopathological parameters investigated. The results demonstrate explicit differences between the activity of matrix metalloproteinase-2 and -9, indicating different roles for both gelatinases in tumour progression. Such data are necessary in order to develop rational anti-cancer therapies based on inhibition of specific matrix metalloproteinases.

Keywords: matrix metalloproteinase; colorectal cancer; gelatin zymography; gelatinase activity assay

Proteinase activity is an important feature in a multitude of physiological and pathological processes. Proteinases are normally strictly regulated in order to prevent undesired degradation of tissue components. In pathological processes like arthritis, periodontal disease, liver fibrosis, cardiovascular disease and cancer, the regulation of proteinase activity is disturbed (Nagase and Woessner, 1999). In cancer, degradation of the extracellular matrix is a key event in tumour cell invasion and metastasis. Various members of the matrix metalloproteinase (MMP) family have been shown to play an important role in these processes, for instance by facilitating the entry of tumour cells into the bloodstream, angiogenesis, tumour cell establishment and growth (Curran and Murray, 2000). The assay methods used vary widely and include analysis of mRNA, protein content and actual proteinase activity. In colorectal cancer (CRC) most of the data are on mRNA, which is upregulated in tumour cells and/or tumour stroma, if compared to normal healthy tissue. For instance, upregulation of mRNA has been reported for MMP-2 (Pyke et al, 1993), MMP-7 (Mori et al, 1995), MMP-9 (Zeng et al, 1996), MMP-11 (Thewes et al, 1996), MMP-12 (Yang et al, 2001) and MMP-14 (Sardinha et al, 2000). In the immunohistochemical detection of MMPs the focus has been on the analysis of MMP localisation. Correlations have been described between MMP-1 immunoreactivity and survival (Murray et al, 1996) and also between stage of disease and immunoreactivity for MMP-1 (Shiozawa et al, 2000), MMP-2 (Levy et al, 1991) and MMP-7 (Adachi et al, 1999). These studies measure MMP immunoreactivity without discrimination between latent proenzyme and activated enzyme. Ultimately, quantitative data on proteinase activity are most relevant for determining the biological role of MMPs in tumour progression.

Quantitative gelatin zymography allows the simultaneous measurement of the latent and active forms of the gelatinases MMP-2 and MMP-9. In recent years, a limited number of studies used zymography to investigate the presence of the gelatinases in tissue samples collected from patients operated for CRC. To some degree, all show enhanced gelatinase levels in tumour tissue, at least for the latent forms of MMP-2 and MMP-9. However, some studies (Liaabakk et al, 1996; Parsons et al, 1998) have not quantified active MMP-9, while others (Garbett et al, 1999a; Zeng et al, 1999b; Baker et al, 2000) do not report specific activities but rather the absence or presence of the various enzyme forms. A consistent quantification of both the latent and active forms – and their ratio – of both gelatinases in matched tumour and normal tissue
is still lacking. Here, we supply these data on samples from a series of CRC patients, which is also large enough to investigate correlations with clinicopathological parameters. In a number of cases we have also measured activities in transitional tissue containing the invasive edge. In addition to quantitative gelatin zymography, we have used a recently developed broad-spectrum bioassay MMP activity using an internally quenched fluorogenic peptide substrate.

MATERIALS AND METHODS

Patients and tissue samples

Seventy-three patients with CRC were included in this study. The paired samples of tumour tissue (n=73), transitional tissue (n=33) and distant normal mucosa (n=73) were collected immediately after surgical resection for primary CRC. Macroscopically vital tumour tissue from the protruding luminal part of the tumour was harvested by a pathologist. Transitional tissue contained the invasive edge on the luminal side including both tumour and normal tissue. Normal colonic mucosa samples were taken at least 5 cm away from the tumour.

Randomly selected samples were analysed histologically to confirm their cellular content. Samples of patients that received pre-operative chemo- or radiotherapy were excluded from the study. All patients provided informed consent and the study was approved by the institutional ethical committee. The patients’ clinicopathological characteristics were determined and used to compose various groups for comparison (cf. Table 2). Tumours were designated mucinous when significant parts of the tumour contained clusters of enlarged mucin producing cells. Tumour staging was according to the current TNM classification, stage I (T1-2N0M0), II (T3-4N0M0), III (TxN1-3M0) and IV (TxNxM1) corresponding to Dukes’ stage A, B, C and D, respectively.

Tissue samples were stored in liquid nitrogen and mechanically disaggregated using a Micro-Dismembrator (Braun), yielding homogenous samples. The pulverised samples were extracted (40 µl per mg tissue) in buffer (0.5 M Tris, 0.2 M NaCl, 10 mM CaCl$_2$ and 1% Triton X-100) for 15 min and freeze–thawed twice. After centrifugation (18 000 r.p.m., 30 min, 4°C) the supernatant was dialysed twice (20 h, 4°C) against a buffer containing 5 mM CaCl$_2$, 30 mM Tris-HCl and 0.2 mM NaCl to remove Triton and the excess of salt. Tissue extracts were stored at −80°C until use.

Protein determination

Protein content was determined in tissue extracts using the bicinchoninic protein assay from Pierce (Rockford, IL, USA). To each extract of 20 µl, 130 µl buffer (50 mM Tris, 0.2 mM NaCl, 5 mM CaCl$_2$, pH 7.5) and 2 ml of bicinchoninic acid solution was added. Following incubation for 30 min at 37°C the absorbance was read at 550 nm. Bovine serum albumin was used as a standard.

Gelatin zymography

Gelatin zymography was performed to quantify the presence of both activated and latent forms of the gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in the tissue extracts (100 µl per lane, 10% SDS-polyacrylamide gels, BioRad, USA) to prevent conversion of TNO211-F by proteinases other than MMPs. Further improvement of the assay specificity for MMPs was achieved by determining the difference in substrate conversion in presence and absence of synthetic MMP inhibitor BB94 (10 µM). The rate of substrate conversion (RFU per µg protein per s) at 30°C (λ$_{ex}$: 485 nm, λ$_{em}$: 530 nm; Cytofluor4000, PerSeptive Biosystems) was normalised to the amount of protein present in the tissue extract and expressed as RFU per µg protein. All samples and reagents were diluted in buffer containing 50 mM Tris (pH 7.5), 5 mM CaCl$_2$, 150 mM NaCl, 1 µM ZnCl$_2$, 0.01% Brij-35 and 0.02% NaN$_3$.

Statistical analysis

Comparisons between MMP levels in matched tumour tissue, transitional tissue and distant normal mucosa were performed using the Friedman two-way ANOVA. As a post-test procedure for pairwise comparisons Dunn’s post-test was used. The relationship between MMP levels and clinicopathological parameters was analysed using the Mann–Whitney U-test when comparing data between two groups; Kruskal–Wallis test was used when comparing more than two groups, with, in-between comparisons using Dunn’s post-test. Differences were considered statistically significant at the P<0.05 level.

Collegenase from Clostridium histolyticum (Type VII, Sigma) was electrophoresed on each gel as an internal standard. After running, the gels were washed three times in 2.5% (v v$^{-1}$) Triton X-100 for 1 min at room temperature to remove SDS. After washing twice in a buffer containing 50 mM Tris-HCl, 5 mM CaCl$_2$ and 0.1% Triton X-100 (pH 7.8), the gels were incubated overnight at 37°C in the same buffer under gentle agitation. Zymograms were stained for 45 min with 0.25% (w v$^{-1}$) Coomassie Brilliant Blue R250 dissolved in 40% methanol and 10% glacial acetic acid. Gels were destained twice for 10 min in the same solution without Coomassie Blue. Proteolytic activities were visualised by clear zones against a dark blue background indicating lysis of gelatin. Loading of the gels was such that proteinase activity was linear with the gelatin lysis. Quantification of the proteinase activities, which were expressed as arbitrary units per mg protein on the basis of size of the lysed area and intensity, was performed using a Sharp JX-330 scanner and Imagemaster ID software (Amersham Pharmacia Biotech, Uppsala, Sweden). In-between comparison of values, obtained on different gels, was performed using the internal standard that was present on each gel. The presence of true MMP activity was confirmed by adding 10 mM EDTA or 1 mM 1,10-phenanthroline, both MMP inhibitors, to the buffers used after electrophoresis. Also human recombinant MMP-2 (1 ng per lane) and -9 (0.5 ng per lane; Oncogene Research Products, Cambridge, MA, USA) were added sometimes to compare their location with endogenous activities (e.g. Figure 1). Activation of recombinant proform MMPs was achieved by incubation with 1 mM p-aminophenylmercuric acetate (APMA).

Fluorometric MMP activity assay

Broad-spectrum MMP activity was determined in 71 out of 73 CRC patient samples (in two cases insufficient sample was left after zymography to perform the activity assay) using the fluorogenic fluorogenic peptide substrate TNO211-F (Dabcyl-Gaba-Pro-Gln-Gly-Leu-Cys(Fluorescein)-Ala-Lys-NH$_2$) essentially as described previously (Beekman et al, 1999; DeGroot et al, 2001). The substrate is mainly cleaved by the gelatinases (MMP-2 and -9) and collagenase 3 (MMP-13) and to a lesser extent by collagenase 2 (MMP-8) and membrane-bound metalloproteinase (MT1-MMP). As such, conversion of this substrate mainly reflects gelatinolytic activity. In short, substrate hydrolysis by tissue extracts was determined in the presence of EDTA-free general proteinase inhibitor Complete (Roche Molecular Biochemicals, Indianapolis, IN, USA) to prevent conversion of TNO211-F by proteinases other than MMPs. Further improvement of the assay specificity for MMPs was achieved by determining the difference in substrate conversion in presence and absence of synthetic MMP inhibitor BB94 (10 µM). The rate of substrate conversion (RFU per µg protein per s) at 30°C (λ$_{ex}$: 485 nm, λ$_{em}$: 530 nm; Cytofluor4000, PerSeptive Biosystems) was normalised to the amount of protein present in the tissue extract and expressed as RFU per µg protein. All samples and reagents were diluted in buffer containing 50 mM Tris (pH 7.5), 5 mM CaCl$_2$, 150 mM NaCl, 1 µM ZnCl$_2$, 0.01% Brij-35 and 0.02% NaN$_3$.
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1. INTRODUCTION

In colorectal cancer (CRC), matrix metalloproteinases (MMPs) play a crucial role in tumour invasion and metastasis. MMPs are a family of zinc-dependent endopeptidases that degrade extracellular matrix components, allowing tumour cells to invade surrounding tissue. The activity of MMPs can be quantified by gelatin zymography, which detects the proteolytic activity of MMPs in tissue extracts.

2. MATERIALS AND METHODS

Seventy-three paired tissue samples of CRC tumour tissue and matching normal mucosa were extracted and analysed by quantitative gelatin zymography. MMP-2 (proMMP-2, active MMP-2) and MMP-9 (proMMP-9, active MMP-9) levels were measured in tumour and normal tissue. The ratio of active to proenzyme was calculated for each sample.

3. RESULTS

Proteinase activity by gelatin zymography

Figure 1 depicts the proteolytic activity of MMP-2 and MMP-9 in CRC tissue extracts detected by gelatin zymography. Active MMP-2 and MMP-9 were not detectable in six and four tissue samples, respectively, whereas the active form of MMP-2 was detectable in the remaining samples.

Table 1: Tumour to normal ratio of MMP activity in CRC

| Type          | n  | Median | Range        |
|---------------|----|--------|--------------|
| Active MMP-2  | 67 | 8.6    | 0.0–947.4    |
| ProMMP-2      | 73 | 1.7    | 0.3–13.1     |
| Active MMP-9  | 69 | 2.0    | 0.0–351.9    |
| ProMMP-9      | 73 | 4.0    | 0.1–35.2     |

MMP activity is expressed in arbitrary units per mg protein. *Patient data were excluded when activity in normal mucosa equaled zero.

4. DISCUSSION

The results indicate a significantly higher level of active MMP-2 in tumour tissue compared to normal mucosa, with statistical significance reached for proMMP-2. ProMMP-9 levels showed a significant correlation with tumour location. Recurrence tumours showed the lowest levels of proMMP-9 whereas the rectum showed the highest levels.

5. CONCLUSION

The data suggest a role for MMPs in CRC progression, with active MMP-2 and MMP-9 playing a crucial role in tumour invasion. Further studies are needed to understand the mechanisms underlying MMP activity in CRC.

Figure 1: Proteolytic MMP activity in human CRC tissue extracts detected by quantitative gelatin zymography. MMP-2 (proMMP-2, active MMP-2) and MMP-9 (proMMP-9, active MMP-9) levels were measured in tumour and normal tissue. The ratio of active to proenzyme was calculated for each sample.

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Correlation between MMP activity and clinicopathological parameters

The relations between clinicopathological parameters and the levels of active and proform MMP-2 and -9 in tumour tissue are shown in Table 2. A number of correlations was found for MMP-2 but only one for MMP-9. ProMMP-9 levels showed a significant correlation with tumour location. In recurrences, tumours showed the lowest levels of proMMP-9 whereas the rectum showed the highest levels.

Table 2: Correlation between MMP activity and clinicopathological parameters

| Parameter | r  | P-value |
|-----------|----|---------|
| Age       | -0.05 | 0.05 |
| T stage   | 0.005 | 0.0005 |
| Node      | 0.010 | 0.0005 |

The absolute activity of both active MMP-2 and -9 was lower in the TxNxM1-group than in the TxNxM0-group.
Comparing the MMP activity with stage of disease, a trend was observed towards lower levels of active MMP-2 and proMMP-2 with advanced stage of disease in tumour (P=0.024 and P=0.044 respectively) and in normal mucosa (P=0.019 and NS respectively). This also applied to the MMP-2 active to proenzyme ratio in tumour (P=0.028) and normal mucosa (P=0.002). In detail differences were most explicit between stage II (T3–4NxM0) and stage IV (TxNxM1) tumours for active MMP-2 (Figure 4) and the MMP-2 active/proenzyme ratio (both P<0.05).

Fluorometric MMP activity assay

Nearly all samples were also measured with a broad-spectrum MMP assay, using a substrate, which is mainly recognised by the gelatinases. Figure 5 shows that overall gelatinolytic MMP activity

| Parameter                  | No. of cases<sup>a</sup> | Active | Proform | Ratio a/p<sup>b</sup> | Active | Proform | Ratio a/p |
|----------------------------|--------------------------|--------|---------|-----------------------|--------|---------|---------|
| Sex                        |                          |        |         |                       |        |         |         |
| Male                       | 43                       | 4.4    | 15.5 (9.4)<sup>d</sup> | 0.3      | 4.4    | 38.4   | 0.1     |
| Female                     | 30                       | 3.6    | 13.9 (6.6) | 0.3      | 6.1    | 46.1   | 0.1     |
| P                          | 0.797                    | 0.622 (0.011)<sup>*</sup> | 0.893    | 0.205 | 0.262 | 0.638 |
| Age                        |                          |        |         |                       |        |         |         |
| <50                        | 12                       | 3.6    | 11.5    | 0.3      | 3.8    | 39.5   | 0.1     |
| 50–70                      | 39                       | 4.3    | 15.4    | 0.3      | 5.1    | 45.0   | 0.1     |
| >70                        | 22                       | 4.1    | 14.8    | 0.3      | 5.7    | 38.9   | 0.1     |
| P                          | 0.747                    | 0.575 | 0.701   | 0.722 | 0.648 | 0.42  |
| Tumour location            |                          |        |         |                       |        |         |         |
| Right                      | 35                       | 3.7    | 12.3    | 0.4      | 5.1    | 43.8   | 0.1     |
| Left                       | 8                        | 4.0    | 13.9    | 0.3      | 11.3   | 125.5  | 0.1     |
| Sigmoid                    | 21                       | 4.0    | 14.4    | 0.3      | 5.5    | 43.3   | 0.1     |
| Rectum                     | 9                        | 5.2    | 15.6    | 0.4      | 2.1    | 27.2   | 0.1     |
| P                          | 0.871                    | 0.559 | 0.75    | 0.888 | 0.040<sup>*</sup> | 0.538 |
| Tumour size (cm)           |                          |        |         |                       |        |         |         |
| <3                         | 5                        | 3.4    | 18.6    | 0.3      | 2.5    | 24.0   | 0.1     |
| 3–5                        | 29                       | 4.0    | 13.7    | 0.4      | 5.0    | 38.4   | 0.1     |
| >5                         | 38                       | 4.2    | 14.7    | 0.3      | 6.3    | 51.1   | 0.1     |
| P                          | 0.846                    | 0.854 | 0.858   | 0.367 | 0.15  | 0.39  |
| Tumour differentiation     |                          |        |         |                       |        |         |         |
| Moderate                   | 48                       | 4.2    | 14.2    | 0.3      | 5.0    | 46.1   | 0.1     |
| Poor                       | 24                       | 3.8    | 14.2    | 0.3      | 5.6    | 41.9   | 0.1     |
| P                          | 0.971                    | 0.99   | 0.599   | 0.867 | 0.924 | 0.971 |
| Mucinous tumours           |                          |        |         |                       |        |         |         |
| No                         | 49                       | 3.8    | 13.7    | 0.3      | 5.1    | 43.8   | 0.2     |
| Yes                        | 23                       | 5.7    | 15.2    | 0.4      | 6.9    | 66.0   | 0.1     |
| P                          | 0.13                     | 0.458 | 0.156   | 0.381 | 0.182 | 0.966 |
| T stage                    |                          |        |         |                       |        |         |         |
| Tis                        | 1                        | 0.8    | 2.4     | 0.3      | 2.5    | 3.7    | 0.7     |
| T1                         | 4                        | 4.6    | 15.2    | 0.3      | 4.4    | 58.0   | 0.1     |
| T2                         | 16                       | 4.0    | 13.5    | 0.3      | 4.9    | 41.1   | 0.1     |
| T3                         | 42                       | 4.0    | 14.2    | 0.3      | 5.2    | 40.5   | 0.1     |
| T4                         | 10                       | 5.9    | 13.9    | 0.3      | 6.6    | 104.7  | 0.1     |
| P                          | 0.792                    | 0.487 | 0.987   | 0.723 | 0.119 | 0.471 |
| N stage<sup>c</sup>        |                          |        |         |                       |        |         |         |
| TxN0M0                     | 39                       | 5.3    | 15.5    | 0.4      | 5.5    | 45.0   | 0.1     |
| TxN1M0                     | 15                       | 3.7    | 13.2    | 0.3      | 5.2    | 26.8   | 0.2     |
| P                          | 0.39                     | 0.946 | 0.213   | 0.721 | 0.145 | 0.765 |
| Distant metastases         |                          |        |         |                       |        |         |         |
| TxNxM0                     | 54                       | 5.0 (0.6)<sup>e</sup> | 15.5 | 0.4 (0.1) | 5.4 | 43.6 | 0.1 |
| TxNxM1                     | 17                       | 2.4 (0.2)<sup>f</sup> | 10.8 | 0.2 (0.0) | 3.6 | 37.4 | 0.1 |
| P                          | 0.005<sup>*</sup> (0.011)<sup>*</sup> | 0.009<sup>*</sup> | 0.007<sup>*</sup> (0.002)<sup>*</sup> | 0.115 | 0.293 | 0.153 |
| Stage of disease           |                          |        |         |                       |        |         |         |
| I                          | 15                       | 5.3 (0.7)<sup>e</sup> | 16.0 | 0.4 (0.1) | 5.8 | 44.0 | 0.1 |
| II                         | 23                       | 6.3 (0.9)<sup>e</sup> | 15.5 | 0.4 (0.1) | 5.5 | 53.0 | 0.1 |
| III                        | 16                       | 4.1 (0.3)<sup>e</sup> | 15.5 | 0.3 (0.0) | 5.9 | 28.8 | 0.2 |
| IV                         | 17                       | 2.4 (0.2)<sup>e</sup> | 10.8 | 0.2 (0.0) | 3.6 | 37.4 | 0.1 |
| P                          | 0.024<sup>*</sup> (0.019)<sup>*</sup> | 0.044<sup>*</sup> | 0.028<sup>*</sup> (0.002)<sup>*</sup> | 0.408 | 0.296 | 0.422 |

* Mann–Whitney U-Test for comparison of two groups; Kruskal–Wallis Test for comparison of non-parametric data of more than two groups. **Only evaluable cases were included. <sup>a</sup>Ratio active/proMMP. <sup>b</sup>Lymph node involvement as a parameter independent of T stage (Tx) excluding distant metastasis (M1). <sup>c</sup>Data between brackets pertain to normal distant mucosa. <sup>d</sup>Activities (arbitrary units per mg protein) are shown in median values. Statistical significant differences are shown with an * (P<0.05).
was significantly higher in both tumour (P < 0.01) and transitional (P < 0.001) tissue than in normal mucosa. In contrast to the MMP-2 activities as determined by zymography, broad-spectrum MMP activity in tumour tissue did not correlate significantly with any of the clinicopathological parameters mentioned in Table 2. The only difference found, was that the transitional tissue of patients with distant metastatic liver disease (n = 5) showed higher activity (3.9 versus 2.0, medians in arbitrary units, P = 0.032) compared to patients without distant metastases (n = 24).

**DISCUSSION**

The present data show that tumour tissue contains higher levels of active and proform MMP-2 and -9 than distant normal mucosa in patients with CRC, with the increase in active MMP-2 being the most pronounced. The active to proform ratio of MMP-2 is highest in tumour tissue, whereas this ratio for MMP-9 is highest in distant normal mucosa. Furthermore, MMP-2 levels do inversely correlate with stage of disease.
The level of proteinase activity in this study was measured using gelatin zymography and broad-spectrum MMP substrate hydrolysis. Zymography separates the proforms and active forms of MMPs but the technique will not distinguish between free MMPs and those complexed with their natural inhibitors, the TIMPs (Kleiner and Stetler Stevenson, 1994). Zymography, therefore, yields no absolute values on the levels of active and latent MMPs in vivo, but rather a representation of the levels of the active and latent forms in both free and complexed form in the various tissues studied. The results should always be interpreted keeping this in mind. The broad-spectrum MMP activity assay, however, does not measure TIMP-complexed MMP activity. Since levels of TIMPs have not been determined in this study, one could speculate that the increased levels of MMP-2 and -9 in tumour will have no actual biological consequence in the in vivo situation because of endogenous inhibition.

However, in a recent study it is shown that the level of TIMP-2 protein, when measured with ELISA, is significantly lower in colorectal tumours than in normal mucosa (Baker et al, 2000). With the high levels of active MMP-2 present in tumour, total inhibition seems therefore unlikely. Moreover, using the MMP activity assay with a fluorogenic peptide substrate, enhanced MMP activity was observed in tumour tissue. Since this assay would not measure MMP activity if all MMPs were inhibited by TIMPs this indicated that indeed the increased MMP activation is not compensated for by increased TIMP production. Also, the ability of TIMP-1, which is reported to be upregulated (Baker et al, 2000), to bind with all MMP-9, can be disputed. TIMP-1 availability should prevent formation of MMP-9 dimers (Goldberg et al, 1992), while we found high levels of the homodimer of MMP-9 (220 kDa, Figure 1) and the heterodimer of MMP-9 and NGAL (135 kDa) (Zeng et al, 1999), indicating that there is unbound MMP-9 left in tumour tissue.

Gelatin zymography demonstrates that both active and proMMP-2 and -9 levels are elevated significantly in primary CRC tissue compared to normal mucosa. These observations are consistent with previous observations on active and proform MMP-2 and proMMP-9 (Liabakk et al, 1996). The presence of active MMP-9 has also been described before (Yamagata et al, 1991; Garbett et al, 1999a; Zeng et al, 1999; Baker et al, 2000), but quantitative measurement in all samples had not been reported up until now. Active MMP-2 increases more than its proform in tumour tissue. Thus there is not only more MMP-2 present in tumour than in normal tissue, but the ratio active to proenzyme also increases substantially. A similar finding was reported by Parsons et al (1998) and McKerrow et al (2000) and supports the view that the activation of MMP-2 is a crucial step in tumour invasion. Expression of MMP-2 mRNA in tumour has been described in stromal fibroblastic cells (Poulsom et al, 1992; Newell et al, 1994), but MMP-2 protein appears to be localised predominantly in the cytoplasm of tumour cells (Ring et al, 1997). A cell membrane-bound metalloproteinase (MT1-MMP), present on tumour cells, has been shown to activate proMMP-2 (Kikuchi et al, 2000) and is most likely responsible for anchoring MMP-2 to these malignant cells. Overexpression of MT1-MMP and enhanced MMP-2 protein activity may thus be induced in the process of tumour progression.

In contrast to MMP-2, the proform of MMP-9 increases much more than active MMP-9 during normal to tumour conversion. This finding is new since previous studies reported difficulty in measuring the active form, because of its unstability (Parsons et al, 1998) or presumed low levels (Liabakk et al, 1996). In CRC it has been shown that MMP-9 mRNA is expressed in macrophages especially in the invasive site. Immunoreactivity has been shown in macrophages and neutrophils but not in tumour cells (Nielsen et al, 1998) and is most likely responsible for anchoring MMP-2 to these malignant cells. Overexpression of MT1-MMP and enhanced MMP-2 protein activity may thus be induced in the process of tumour progression.

**Figure 4** Active MMP-2 in primary CRC tissue extracts of stage I (n=15), II (n=23), III (n=16) and IV (n=17) tumours determined by quantitative gelatin zymography. Horizontal bars represent median values. Stage II versus stage IV: P < 0.05.

**Figure 5** Broad-spectrum MMP activity in CRC tumour (n=71), transitional (T – N; n=29) and normal tissue (n=71) determined by quenched fluorogenic substrate hydrolysis. Activity is expressed as the rate of substrate conversion on basis of protein. Horizontal bars represent median values, boxes represent the interquartile range, vertical lines represent the 10 to 90% range of the observations. *P < 0.01, **P < 0.001 versus normal mucosa.

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Comparison between clinicopathological parameters and gelatinase activity reveals correlations for MMP-2, in particular in relation to stage of disease. Neither Liabakk et al. (1996) nor Parsons et al. (1998) found such a correlation, but these authors have not included stage IV tumours in their studies. Indeed, in our series activities are found in stage I, II and III tumours similar to those of Parsons et al. (1998), but a significantly lower activity of both active and proMMP-2 is found in metastatic stage IV tumours if compared to all tumours without distant (c.q. liver) metastases. It has been suggested that MMP activities in the separate disease stages may vary because of differences in stromal components surrounding the tumours (Liabakk et al., 1996). Although this seems reasonable in view of the fact that different cell – cell contacts and specific cell – matrix interactions may cause changes in MMP expression (DeClerck, 2000), one would then also expect variations in MMP activity with depth of invasion. However, we find no correlations between MMP activity and T stage. The finding that active MMP-2 in normal mucosa is also inversely correlated with stage of disease and with distant metastatic disease is remarkable. Although the activities of active MMP-2 were much lower, they show a similar trend. This may be due to the effect of local diffusion or perfusion via lymph or blood vessels. Previous studies on MMP activities have not compared normal tissue MMP activities with clinicopathological parameters, most probably due to difficulties measuring these low activity levels.

The observation that in normal mucosa the level of proMMP-2 is higher in males than in females has not been described before. Its relevance in cancer is doubtful because these differences are seen in normal tissue only, where activities are very low, and do not pertain to active MMP-2. Also, the correlations between proMMP-2 levels and other clinicopathological parameters do not result from a disbalance in gender.

The correlation between tumour proMMP-9 levels and colonic location has not been found before. Zeng et al. (1996) did not find correlation between MMP-9 mRNA expression and location. This probably means that the increased presence of the proenzyme is caused by upregulation at the translational level. While no further significant correlations between MMP-9 activity and clinicopathological parameters do not result from a disbalance in gender.

The correlation between tumour MMP (production and) activation is elevated compared to the production of TIMPs. This results in an excess of free, active MMPs, which may contribute to the invasiveness of the tumour.

In summary, our results show increased levels of MMP activity in tumour and transitional tissue. A variety of MMPs may be responsible for this observation since the substrate is hydrolysed by all MMPs that exhibit gelatinolytic activity (e.g. MMP-2, -9 and -13). By the use of a specific MMP inhibitor as a control in this activity assay and the use of a general protease inhibitor, only actual active MMPs are measured and not other proteinases or protein-inhibitor complexes. This is an important difference with assays used by others (Garbett et al., 1999b; Baker et al., 2000; McKerrow et al., 2000). The results obtained here indicate that in the tumours studied MMP (production and) activation is elevated compared to the production of TIMPs. This results in an excess of free, active MMPs, which may contribute to the invasiveness of the tumour.

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