Proteolysis in colorectal cancer

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Abstract
Background—The process of metastasis is complex, involving many interrelated stages, including proteolysis. Proteolysis occurs in both normal and pathological processes and involves the breakdown of the extracellular matrix and/or basement membrane by proteolytic enzymes. Normally, proteolysis is tightly controlled by specific endogenous proteinase inhibitors. However, in certain disease processes, including cancer, controlled but abnormal proteolysis seems to occur. Proteinases involved in tumour invasion and metastasis include the matrix metalloproteinases (MMPs) and the serine proteinases.
Aims—To gain a greater understanding of the proteolytic process occurring in colorectal cancer and to determine which, if any, proteinases are upregulated.
Methods—The synthesis of proteinases and their inhibitors was compared in paired tumour and normal tissue samples from patients with colorectal cancer (n = 24). Substrate zymography was used to determine the synthesis of MMPs (MMP-2, MMP-9, and MMP-3) and the plasminogen activators (urokinase and tissue-type plasminogen activators); enzyme linked immunosorbent assays (ELISAs) were used to determine the concentrations of MMP-1 and tissue inhibitor of metalloproteinase 1 (TIMP-1); and the technique of quenched fluorescence substrate hydrolysis was performed to determine the total MMP activity of each sample.
Results—In general, both proteinase and inhibitor expression was greater in the tumour tissue when compared with the corresponding normal colorectal tissue. The amount of active MMPs was greater in the tumour tissue.
Conclusions—The increased extracellular proteinase concentrations and activity may encourage tumour invasion and metastasis. This study points to MMP-9 as being of potential major importance in the development of this form of cancer.
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Keywords: colorectal cancer; metastasis; proteolysis

The process of metastasis is complex, involving many sequential and interrelated steps. In brief, metastasis involves detachment of a single or a group of tumour cells from the primary tumour, local invasion through the surrounding extracellular matrix (ECM), intravasation into blood or lymphatic vessels, survival in the circulation, extravasation at a secondary site, survival and proliferation at the secondary site, and finally the potential for this secondary growth to metastasise.[1]

ECM turnover normally involves homeostatic control of cell division, matrix synthesis, and degradation (proteolysis), which is under the control of cytokines, growth factors, and cell–matrix interactions. ECM degradation by proteinases is involved in normal physiological processes and is a tightly controlled and regulated process. However, in certain disease states, including cancer invasion and metastasis, the balance of ECM turnover is not controlled or regulated normally and excessive proteolysis occurs.[2]

Proteinases are known to be involved in tumour cell invasion and the resulting ECM degradation may occur at several stages of the metastatic cascade including angiogenesis, local invasion, intravasation, and extravasation. Several proteinases have been implicated in one or more of these processes, as well as being potential prognostic indicators of disease free survival and death; these proteinases include: cathepsin B,[3] cathepsin D,[4] and urokinase-type plasminogen activator (uPA).[5]

The ECM degradation required for tumour cell invasion is a tightly controlled process at the biochemical and cellular level. These processes are dependent not only on the total amount of secreted proenzymes, but also on the coordinated activation and inhibition of these enzymes. The cells often regulate proenzyme activation either on or close to the cell surface, permitting localised ECM degradation.[6]

Several proteinase classes exist including metalloproteinases, serine proteinases, aspartic proteinases, and cysteine proteinases. Within the metalloproteinase class, the matrix metalloproteinase (MMP) or matrixin family of enzymes is thought to be particularly important in ECM turnover. The serine proteinases, plasminogen activators (PAs), are also involved in ECM degradation by activating plasminogen to the proteinase plasmin. Plasmin can degrade ECM components directly or indirectly by activating other proteinases. The role and regulation of the MMP and PA systems have been reviewed previously.[7] [8]

In our study, the expression of several MMPs and serine proteinases was measured in colorectal cancer by means of three different techniques in paired tumour and normal tissue samples. The first technique, substrate zymography, determines the total expression of potentially active MMP-2 (gelatinase A), MMP-3 (stromelysin 1), MMP-9 (gelatinase B), uPA, and tissue type PA (tPA). Second, enzyme linked immunosorbent assays (ELISAs) were used to determine MMP-1

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and tissue inhibitor of metalloproteinase 1 (TIMP-1) protein concentration. Finally, quenched fluorescence substrate hydrolysis was used to determine the overall MMP activity within the colorectal tissues, identifying whether there is an imbalance in MMP and TIMP synthesis favouring proteolysis.

Previous studies have determined the expression of MMPs,[9–20] PAs,[21–23] and TIMPs[12 13 24 25] in colorectal cancer; however, most studies have involved only a single technique and have looked at individual proteinases.

Materials and methods

MATERIALS
Acrylamide/bis acrylamide, ammonium persulphate, calcium chloride, Coomassie blue R-250, gelatin, glycine, lauryl sulphate (sodium dodecyl sulphate, SDS), sodium chloride, N, N, N'-tetramethylethylenediamine (TEMED), trizma base and wide range molecular weight marker were purchased from Sigma (Poole, Dorset, UK), and Triton X-100 was purchased from BDH (Glasgow, Scotland, UK). MMP protein standards were purchased from TCS Biologicals (Buckingham, Buckinghamshire, UK). MMP-1 and TIMP-1 ELISAs, the coumarin labelled peptide used as a substrate for MMPs, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$ and the standard Mca-Pro-Leu-OH were obtained from Calbiochem (Nottingham, Nottinghamshire, UK).

TISSUE SAMPLES
Fresh paired tumour and normal colorectal tissue samples (n = 24) were collected in RPMI media by a consultant pathologist from the histopathology department, Royal Hallamshire Hospital, Sheffield after surgical resection for colorectal cancer. The tissue samples were disaggregated mechanically, using scalpel blades and graded needles, to yield a single cell suspension. The cell suspension was centrifuged at $750 \times g$ for 10 minutes. The cell pellet was resuspended and the viable cells were counted using a haemocytometer (Neubauer; Phillip Harris Scientific, Blyth, Northumberland, UK). After counting, the cell pellet was reformed and the cells resuspended in lysis buffer (0.1% Triton X-100 in 0.05 M trizma base, 0.2 M NaCl, and 0.005 M CaCl$_2$) at a concentration of 10×10^6 cells/ml buffer.

ZYMOGRAPHY
Gelatin zymography
The tissue sample lysates were diluted 3/1 with non-reducing sample buffer (0.5 M Tris/HCl, pH 6.8, SDS, glycerol and bromophenol blue).

Gelatin zymography was performed to determine the presence of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in the tissue samples.[26] Each sample (20 μl) was run in parallel with a molecular weight marker and MMP-2 and MMP-9 protein standards (where available) on an SDS polyacrylamide gel (12%) containing 0.1% gelatin as the substrate, at 200 V for one hour (mini-V 8.10 BRL; Life Technologies, Paisley, Scotland, UK). After electrophoresis, the gel was washed in 2% Tri-

ton X-100 for one hour at room temperature on an orbital shaker. The substrate gel was then incubated overnight with MMP incubation buffer (0.05 M trizma base, 0.2 M NaCl, 5 mM CaCl$_2$, pH 7.4). After incubation, gels were stained with a 0.2% solution of Coomassie blue for 15 minutes and then destained (10% acetic acid and 30% methanol) for 10 minutes. Proteolytic activity was represented by clear lysis bands of degraded protein on a uniformly blue background.

Casein zymography
The presence of MMP-3 (stromelysin-1) was determined using a 12% SDS polyacrylamide substrate gel containing 0.1% casein.[27]

Control gels for MMPs
Control gels contained either of the MMP inhibitors, 10 mM EDTA or 1, 10-phenanthroline in the MMP incubation buffer to confirm that the lysis bands were the result of MMP activity.

Double substrate zymography
Double substrate zymography was used to determine the presence of PAs in tissue samples.[27] The two substrates used were plasminogen (substrate 1) and gelatin (substrate 2). Plasminogen acts as a substrate for any PAs present in the sample, by cleaving plasminogen to the active enzyme, plasmin, which subsequently degrades the gelatin. The PA incubation buffer (0.25 M trizma base, pH 8.1) contained 10 mM EDTA to eliminate any gelatinase activity in the sample.

Control gels for PAs
Each sample was also run on two control gels. The first gel contained only gelatin as a substrate; therefore, any PAs present in the samples would be unable to degrade the gelatin. The second gel contained the serine proteinase inhibitor phenylmethylsulphonyl fluoride (PMSF) in the incubation buffer to determine whether lysis bands were the result of the activity of PAs.

Quantitation of the gels
Gels were quantitated using laser densitometry. Gels were scanned and analysed using the Quantity One software package (Discovery Series; Pharmacia Biotech, Milton Keynes, Buckinghamshire, UK). The image of the gel was inverted to reveal dark bands on a white background. The molecular weight, area, and optical density of each band were determined. The relative proteinase activity was determined for each proteinase by multiplying the area of each band by its optical density.

ELISA
MMP-1 and TIMP-1 concentrations were also measured in colorectal tissue samples by means of commercially available ELISA kits (MMP-1: Amersham Life Sciences, Amersham, Buckinghamshire, UK; TIMP-1: Calbiochem, Nottingham, UK). Absorbance was measured at 450 nm and a calibration curve drawn. The concentration of antigen in each sample was
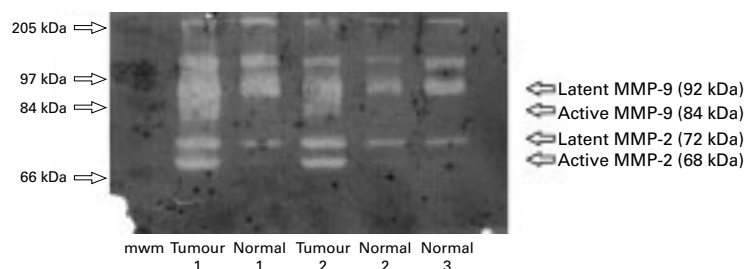
Figure 1   Gelatin zymogram illustrating the gelatinolytic activity of two paired colorectal tumour and normal tissue samples. The first lane contains the molecular weight markers (mwm). Latent and active forms of matrix metalloproteinase 2 (MMP-2) and MMP-9 were detected in both tumour samples; however, only the latent forms of these enzymes were detected in the normal samples.
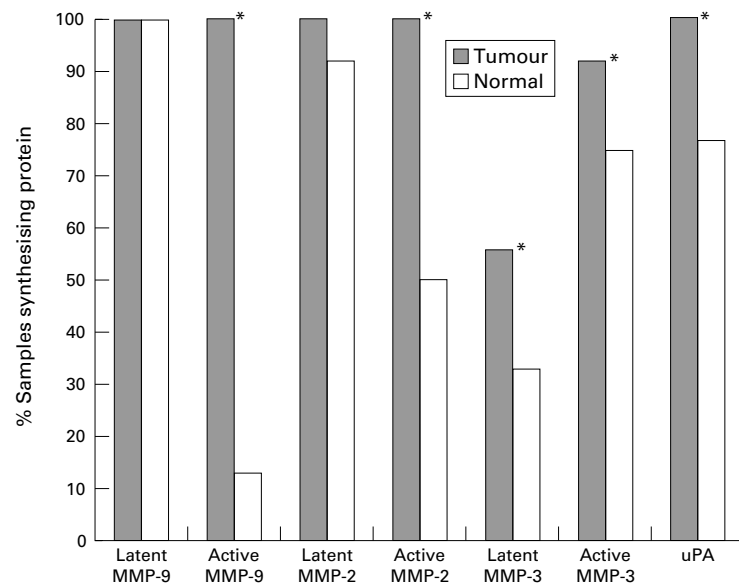

Figure 2   Graph showing the percentage of colorectal tissue samples (tumour and normal synthesising both latent and active forms of the enzymes indicated (*p < 0.05; χ² test). MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator.

then determined using a Dynatech plate reader connected to Revelation software (Dynatech Laboratories, Billingshurst, West Sussex, UK).

QUENCHED FLUORESCENCE SUBSTRATE HYDROLYSIS

The technique of quenched fluorescence substrate hydrolysis uses the quenched fluorescence substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, which is cleaved by all secreted activated MMPs tested so far[28] at the Gly-Leu bond, releasing the fluorescent Mca group from the internal quenching group Dpa.[29] The total MMP activity was determined in colorectal tumour and normal tissue samples by incubating 150 μl tissue sample lysate with 2835 μl assay buffer (0.1 M Tris/HCl, 0.1 M NaCl, 10 mM CaCl₂, pH 7.5) and 15 μl of the fluorescent substrate (5 μM). The samples were incubated for three hours at 37°C and the MMP activity was determined on a fluorimeter (Perkin Elmer LS50B; Perkin Elmer, Beaconsfield, Buckinghamshire, UK) (λ_{ex} 328 nm and λ_{em} 393 nm) running the FLDM software. Lysis buffer (150 μl) incubated as above acted as the negative control.

The fluorimeter was standardised (maximum fluorescence was set by the addition of 0.5 μM Mca-Pro-Leu-OH) so that the abso-

lute rate of substrate hydrolysis was determined for each sample. The rate of substrate hydrolysis was compared between samples and expressed as pM/minute.

STATISTICAL ANALYSIS

For comparisons between proteinase and inhibitor expression in colorectal tumour and normal tissue samples the Mann Whitney U test for non-parametric data, with 95% confidence limits was performed. For comparisons between the number of samples synthesising each proteinase, the χ² test was performed. The data were considered to be significant at the p < 0.05 level. The number of patients studied was not sufficient to justify comparisons of proteinase and inhibitor expression between the stage/grade of tumours.

Results

PROTEINASE CONCENTRATIONS MEASURED BY SUBSTRATE ZYMOGRAPHY

MMP-2 and MMP-9

After gelatin zymography, a maximum of six lysis bands was observed, with molecular masses of 205 kDa, 116 kDa, 92 kDa (latent MMP-9), 84 kDa (active MMP-9), 72 kDa (latent MMP-2), and 68 kDa (active MMP-2). Figure 1 shows a representative gelatin zymogram illustrating the gelatinolytic activity of two paired colorectal tumour and normal tissue samples.

The 72 kDa band corresponding to latent MMP-2 was present in all colorectal tumour samples and 22 of 24 normal samples (92%). As determined by densitometry, latent MMP-2 expression was significantly greater in 18 of 24 tumours compared with the corresponding normal tissue (p < 0.05). The 68 kDa band corresponding to active MMP-2 was found in all tumour samples but in only half (12 of 24) of normal tissue samples (p < 0.001; fig 2). Active MMP-2 expression was significantly greater in the tumour tissue in 23 of 24 paired colorectal samples studied. Similarly, the lysis band migrating at 92 kDa, corresponding to latent MMP-9, was present in all tumour and normal tissue samples analysed. However, the 84 kDa band corresponding to active MMP-9 was present in all tumours but only in three of 24 normal samples (13%) (fig 2).

MMP-3

After casein zymography, colorectal samples produced two different lysis bands, migrating at the corresponding molecular mass to the latent (57/59 kDa) and active (< 45 kDa) forms of MMP-3. Two other lysis bands were observed in some tissue samples, with molecular masses of > 100 kDa and 84 kDa.

A greater proportion of colorectal tumour samples expressed both latent and active MMP-3 than did normal colon tissue (p < 0.01; χ² test). A lysis band migrating with the latent MMP-3 protein was present in 14 of 24 tumours (56%) and eight of 24 normal tissue samples (33%), and the active MMP-3 lysis band was present in 23 of 24 tumours (92%) and 18 of 24 normal tissue samples (75%) (fig 2). However, there was no signifi-
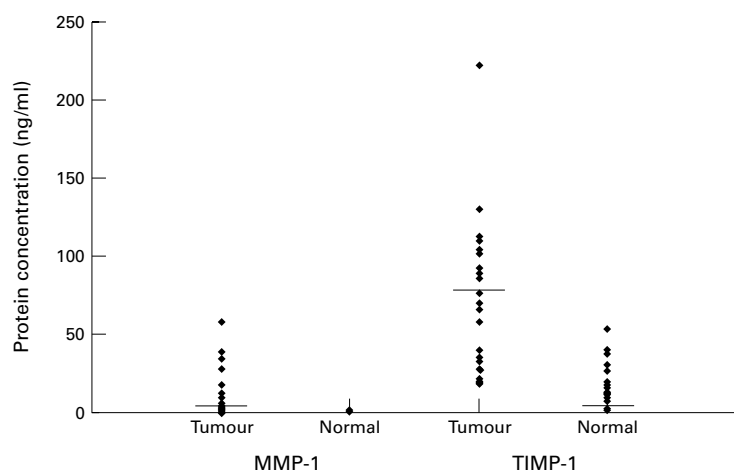
Figure 3   Graph showing matrix metalloproteinase 1 (MMP-1) and tissue inhibitor of metalloproteinase 1 (TIMP-1) concentrations (ng/ml) estimated by enzyme linked immunosorbent assay in paired colorectal tumour and normal tissue samples (n = 24). Both MMP-1 and TIMP-1 concentrations were significantly higher in the tumour tissue (*p < 0.05; Mann Whitney U test).

Figure 4   Graph demonstrating the rate of substrate hydrolysis in paired colorectal tumours and their corresponding normal tissue samples (n = 24). The line respresents the median rate of substrate hydrolysis, with a significantly greater rate being seen in colorectal tumour samples (< 0.05; Mann Whitney U test).

cant difference in the amount of latent and active MMP-3 or the total amount of MMP-3 between tumour and normal tissue samples (results not shown).

None of the tissue samples showed lysis bands after gelatin and casein substrate zymography when control gels were incubated with MMP inhibitors (not shown); therefore, the lysis bands observed on normal gels were a result of the presence of MMPs within the tissue samples.

PAs
The only PA to be found in colorectal tissue comigrated with the 54 kDa uPA control. No lysis band was seen at 70 kDa and therefore no tPA was present in any tissue. This 54 kDa lysis band was found in all tumour tissue samples and 17 of 22 normal tissue samples. In 18 of 22 paired samples, uPA concentrations were greater in the tumour tissue than in the corresponding normal tissue (results not shown; p < 0.05; Mann Whitney U test).

No lysis bands were seen in control gels (gelatin alone or gel incubated with serine proteinase inhibitors). Therefore, the lysis bands seen after double substrate zymography were the result of PAs present in the tissue samples.

ELISA RESULTS
Both MMP-1 and TIMP-1 concentrations were significantly greater in colorectal tumours than the corresponding normal tissues (p < 0.05) as assessed by ELISA. The range of MMP-1 concentrations was from 0.01 to 58.1 ng/ml for tumour tissues and 0.01 to 1.7 ng/ml for normal tissue samples. The median values were 3.8 and 0.2 ng/ml, respectively (fig 3). In 20 of 24 paired samples, MMP-1 concentrations were significantly greater in the tumour tissue than in the corresponding normal colon (p < 0.05). Similarly for TIMP-1, concentrations in tumours ranged from 18.4 to 222 ng/ml and for normal tissue from 1.4 to 53.6 ng/ml, with median values of 76.6 and 12.45 ng/ml, respectively (fig 3). In all paired samples, TIMP-1 concentrations were
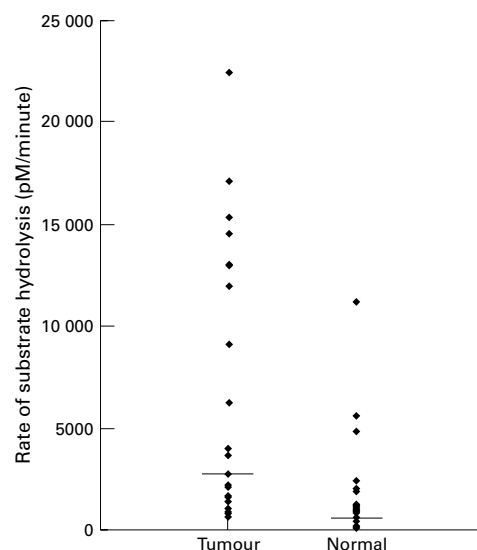
significantly greater in the tumour tissues (p < 0.01).

TOTAL MMP ACTIVITY
All colorectal tumour and normal tissue samples studied had some MMP activity because the quenched fluorescence substrate was cleaved by all tissue samples. However, the rate of substrate hydrolysis varied considerably between samples (fig 4); tumour tissue ranged from 791 to 22 470 pM/min and normal colorectal tissue from 111 to 11 133 pM/min. The median rates were 2208 and 978 pM/min, respectively. In 21 of 24 paired colorectal samples, the rate of substrate hydrolysis was significantly greater for tumour tissue than the corresponding normal tissue (p < 0.05).

Discussion
Previously, the expression of MMPs has been determined in colorectal cancer by several groups, using different techniques including zymography,[11 16] immunohistochemistry,[12 13] ELISA,[14] northern blotting,[15] and in situ hybridisation.[10] Several techniques have also been used to determine PA expression including immunohistochemistry,[23 30] in situ hybridisation,[22] and ELISA.[22] However, no individual study has combined these methods. To gain increased understanding of the role of these proteinases in cancer, we need to know where they are processed (and activated for MMPs), which cells synthesise them, the different forms of each MMP synthesised, and whether the proteinases are complexed with their inhibitors.

Three techniques were used in our study: zymography to determine proteinase expression, ELISA for MMP-1 and TIMP-1 protein concentrations, and quenched fluorescence substrate hydrolysis to determine the total MMP activity within the samples.

Our study describes the presence of multiple proteinases in normal and malignant colorectal tissues. The greatest differences in proteinase synthesis were seen after gelatin zymography, where the expression of the active forms of both MMP-2 and MMP-9 was significantly greater in tumours than in the normal colon. The production of the latent and active forms of MMP-2 and MMP-9 varied between tissues and this might reflect the differences in the levels of secretion, synthesis, and activation of these two gelatinases and, therefore, their involvement at the different stages of tumour progression and metastasis.

The abilities of the gelatinases to degrade gelatin in the substrate gel varied. A higher concentration of MMP-2 was required to yield an equivalent lysis band to that of MMP-9 when calibration curves were constructed for each gelatinase (data not shown).

Previous studies using gelatin zymography on 192[11] and 53[16] biopsies from colorectal carcinomas found a greater expression of latent MMP-9, latent MMP-2, and active MMP-2 in the tumour tissues compared with normal colorectal tissues. However, no mention was made of active MMP-9, which was seen in all colorectal tumour samples in our study.[11 16] Possible explanations for the differential gelatinase expression between studies could be the use of different disaggregation techniques or differences in the area of tumour from which the sample came. In a study correlating MMP expression with Dukes's staging of the tumour, it was noted that both latent MMP-9 and active MMP-2 expression were significantly greater in Dukes's A and C tumours than Dukes's B tumours.[11] It was suggested that the difference in the enzyme activity was the result of differences in the stromal components between Dukes's A and B tumours. Because of the small sample numbers involved (n = 24), no attempt was made to correlate MMP production with the pathological stage in our study.

Other groups have determined the expression of the gelatinases using northern blotting[10 15 17–19] in situ hybridisation,[10 16] immunohistochemistry,[13] and ELISA.[14] The cellular production of the mRNAs and the proteins for the gelatinases, as well as the clinical implications of their synthesis, have been determined. Similar to the results for MMP-9 protein synthesis in our study, Zeng and Guillem found that MMP-9 mRNA expression was greater in colorectal tumours than normal tissues, and that the signal for both the protein and mRNA was strongest in stromal cells at the tumour–stromal interface of an invading neoplasm.[19] By contrast, MMP-2 mRNA expression was found to be strongest in stromal cells, namely endothelial cells and fibroblasts.[17] Using immunohistochemistry, Gallegos and colleagues found MMP-2 on fibroblasts and in the ECM and MMP-9 on macrophages.[13] Prognostically, it was found that increased MMP-9 mRNA expression was associated with shorter disease free and overall survival.[18]

Both latent and active MMP-3 were found in a greater proportion of colorectal tumours than in the surrounding normal tissue, and active MMP-3 was found in a greater proportion of both tissues than was latent MMP-3. The difference between MMP-3 expression observed in colorectal tissue was not as disparate as that seen for MMP-2 or MMP-9. From the large number of normal tissue samples producing some form of MMP-3, it appears that MMP-3 plays some role in the physiology of the normal colon. In previous studies, the synthesis of MMP-3 was detected in the ECM by means of immunohistochemistry,[13] and Matrisian and colleagues found MMP-3 on stromal cells.[9]

All tissue samples synthesised MMP-1, although significantly higher concentrations were found in the tumour tissue than the normal tissue in all paired samples. The involvement of MMP-1 in tumour progression is likely to be in the initial cleavage of the interstitial collagens, enabling other proteinases to complete the degradation of collagen. This specific action of MMP-1 would explain the decreased synthesis of MMP-1 compared with the other MMPs studied. Previously, Murray and colleagues found the expression of MMP-1 to be associated with a poor prognosis.[20] However, in contrast, another study detected no MMP-1 in colorectal tumours using immunohistochemistry.[13]

The only PA found in colorectal tissue was uPA, with significantly more synthesis in tumours than in normal tissue samples, which confirms previous studies,[21 22] and implies a possible role for uPA in colorectal tumour invasion and metastasis. Previous studies have shown uPA mRNA expression to be confined to fibroblasts,[22] and immunohistochemical staining has detected uPA in fibroblasts and endothelial cells[21]; in both cases, greater staining was seen in tumour tissue than in normal colon samples. Prognostically, uPA concentrations have been shown to be significantly higher in Dukes's C than Dukes's A tumours, and the uPA:PAI-1 ratio at the tumour–host interface has been related to tumour aggressiveness.[23]

All tissue samples studied produced TIMP-1. However, synthesis was significantly greater in tumours than the corresponding normal tissue. TIMP-1 synthesis has been shown previously throughout the malignant tumour stroma.[10] However, in contrast, another study found TIMP-1 in only three of 40 patients studied by means of immunohistochemistry. Prognostically, increased TIMP-1 synthesis was shown to correlate with positive lymph node involvement and five year survival.[24] Both TIMP-1 and TIMP-2 synthesis have been shown to correlate with Dukes's classification[24] and TIMP-2 expression has been shown to be a predictor of tumour stage.[25]

Both proteinase and inhibitor synthesis were found to be upregulated in colorectal tumours. However, the most important determinant for proteolysis in vivo is the balance between the synthesis and activation of proteinases and the synthesis of their inhibitors. Quenched fluorescence substrate hydrolysis determined the amount of free active MMPs present within the tissue samples. This is indicative of proteolysis occurring in vivo and has not been determined

previously in colorectal cancer. The total MMP activity was significantly greater in the tumour tissue and this may be relevant to tumour invasion and metastasis in vivo.

Wide variations were seen in the expression of the proteinases and inhibitors studied and in the total MMP activity within and between colorectal tissues. Because the numbers of cells in each sample were the same, any differences observed between samples might be the result of differences in the relative proportions of cells (for example, tumour cells compared with fibroblasts) and the tissue's ability to produce, secrete, and activate proteinases or their inhibitors. Two possible explanations for the differential synthesis between tumour samples are: first, the area of the tumour from which the sample came and, second, the stage or grade of the tumour, which was not determined in our study because of the small sample numbers.

Previous studies have demonstrated the expression of other MMPs not analysed in our study in colorectal cancer, including matrilysin,[31] stromelysin-3,[9] and membrane-type MMP-1.[32] These proteinases may also be partially responsible for hydrolysing the fluorescence substrate and this requires future consideration.

In summary, our results show increased synthesis of some proteinases in tumour tissue when compared with tissue from the normal colon and suggest a possible role for MMP-9, in particular, in relation to colorectal pathology.

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