Tumor development of colon cancer in rat liver
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Chapter 6

*In situ* localization of gelatinolytic activity in the extracellular matrix of metastases of colon cancer in rat liver using quenched fluorogenic DQ-gelatin

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Summary

Matrix metalloproteinases (MMPs) such as gelatinases are believed to play an important role in invasion and metastasis of cancer. In this study we investigated the possible role of MMP-2 and MMP-9 in an experimental model of colon cancer metastasis in rat liver. We demonstrated with gelatin zymography that the tumors contained MMP-2 and MMP-9, but only MMP-2 was present in the active form. Immunolocalization of MMP-2 showed that the protein was localized at basement membranes of colon cancer cells and in intratumor stroma, associated with extracellular matrix (ECM) components. However, zymography and immunohistochemistry (IHC) do not provide information on the localization of MMP activity. Therefore, we developed an in situ zymography technique using the quenched fluorogenic substrate DQ-gelatin in unfixed cryostat sections. The application of DQ-gelatin in combination with a gelled medium allows precise localization of gelatinolytic activity. Fluorescence due to gelatinolytic activity was found in the ECM of tumors and was localized similarly to both MMP-2 protein and collagen type IV, its natural substrate. The localization of MMP-2 activity and collagen type IV at similar sites suggests a role of MMP-2 in remodeling of ECM of stroma in colon cancer metastases in rat liver.

Key words: gelatinase activity, DQ-gelatin matrix, metalloproteinase, metastasis, extracellular matrix.

Introduction

Matrix metalloproteinases (MMPs; e.g., gelatinases, collagenases, stromelysins, matrilysins and membrane-type MMPs) play an important role in invasion and metastasis of cancer (Crawford and Matrisian 1994). These enzymes degrade the extracellular matrix (ECM) (Knittel et al. 2000), enabling cancer cells to migrate and proliferate (Ray and Stetler-Stevenson 1994; Koyama et al. 2000). Expression of MMP-2 and MMP-9 is increased in many tumors compared with normal tissues (Liabakk et al. 1996; Davidson et al. 1999), and activation of the proform of MMP-2 is crucial for the metastatic capacity of primary tumors (Waas et al. 2002). MMP-2 and MMP-9 are considered to be the major MMPs involved in invasion and metastasis because of their capacity to degrade type IV collagen, an important component of basement membranes. Degradation of basement membranes is an essential step in invasive growth and metastasis (Himmelstein et al. 1994; Liabakk et al. 1996; Ratnikov et al. 2000). However, it is becoming appreciated that MMPs are not only involved in the
degradation of matrix components. In addition to collagen type IV, a number of soluble growth factors, growth factor receptors, and cytokines are also natural substrates for MMP-2 and MMP-9 (Gearing et al. 1994; Schonbeck et al. 1998; Yu and Stamenkovic 2000). Soluble growth factors and cytokines are secreted and can be "stored" bound to ECM components. On matrix remodeling, these molecules are liberated and activated or inactivated, giving rise to a local increase or decrease in bioactive molecules (McCawley and Matrisian 2000). Furthermore, processing of growth factor receptors by MMP-2 or MMP-9 can activate or inactivate their biological function (Levi et al. 1996; Lombard et al. 1998). Immunohistochemical (IHC) studies demonstrated that MMP expression is not restricted to cancer cells but can also be found in stromal cells of tumors (Meyer and Hart 1998; Baker et al. 2000).

MMP inhibitors, such as batimastat and marimastat, are currently used to inhibit cancer growth and metastasis in animal models and have been tested in Phase III clinical trials (Rasmussen and McCann 1997; Prontera et al. 1999). Treatment with batimastat reduced the vascular density of metastases in mice and therefore appeared to inhibit angiogenesis (Wylie et al. 1999). On the other hand, liver metastases were also induced by batimastat, which was in accordance with upregulation of MMP-2 and MMP-9 mRNA levels in these livers (Kruger et al. 2001). In line with these experimental data, synthetic MMP inhibitors have been shown to exert dual effects in clinical trials. Marimastat treatment increased progression-free survival in patients with inoperable gastric cancer, but in advanced pancreatic cancer no benefit was found in comparison with the current standard treatment with gemcitabine. Bayer Pharmaceuticals (West Haven, CT) halted all clinical trials of its MMP inhibitor BAY 12-9566 because, in a trial of small-cell lung cancer patients, the inhibitor did worse than the placebo (McCawley and Matrisian 2000). These contradictory findings indicate the complexity of the involvement of gelatinases in tumor progression. Therefore, the success of treating cancer patients with MMP inhibitors is largely dependent on our understanding of the molecular mechanisms involved.

A model of metastasis of colon cancer cells in rat liver has been used in our group to study processes involved in metastasis (Jonges et al. 1993; Griffini et al. 1997). With respect to the role of proteases, we found that inhibitors of extracellular cathepsin B delayed rather than inhibited colon cancer metastasis in rat liver. We concluded that active extracellular cathepsin B was involved in the development of these tumors but that its function was not an essential
requirement (Van Noorden et al. 1998). Therefore, we are now investigating whether MMP-2 and MMP-9 activity is essential in the development of these metastases. Because MMP activity is highly regulated by activators of proforms, mostly other proteases, as well as by endogenous inhibitors, such as tissue inhibitors of matrix metalloproteinases (TIMPs; Koyama et al. 2000), localization of MMPs with specific antibodies does not provide information on the activity of these enzymes. On the other hand, gelatin zymography, a technique that is frequently used to demonstrate MMP-2 and MMP-9 activity in tissue homogenates after polyacrylamide gel electrophoresis (Heussen and Dowdle 1980; Creemers et al. 1998), does not allow conclusions to be drawn with respect to localization of activity. *In situ* zymography enables localization of activity in unfixed cryostat sections. This principle was introduced by Galis et al. (1995) using FITC-labelled gelatin, which allows the localization of enzyme activity in tissue sections. However, this technique resulted in poor localization of gelatinase activity and quantification was not possible, mainly because decreases in fluorescence rather than production of fluorescence must be detected.

In this study we investigated the possible involvement of MMP-2 and MMP-9 activity in experimentally induced colon cancer metastases in rat liver using gelatin zymography and IHC. Moreover, we demonstrate a technique for specific and sensitive localization of gelatinolytic activity in unfixed cryostat sections of rat liver containing metastases using DQ-gelatin as fluorogenic substrate. We show that DQ-gelatin, which was introduced for the fluorometric determination of gelatinolytic activity in vitro, is an excellent substrate for visualization of gelatinolytic activity *in situ*.

**Materials and Methods**

**Animals**

Adult male WagRij rats (Broekman; Someren, The Netherlands) with a body weight of 200–250 g were maintained for 2 weeks under constant environmental conditions with free access to food and water. All animal experiments were performed in agreement with the Animal Ethics Committee of the Academic Medical Center, University of Amsterdam.
Cancer cells

An established colon carcinoma cell line, CC531s, was developed by Marquet et al. (1984). The cells were cultured at 37°C as monolayers in RPMI-1640 Dutch Modification without 1-glutamine (GIBCO/BRL; Grand Island, NY) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 IU penicillin/ml, and 100 mg streptomycin/ml. Cells were washed with PBS and, after detachment with trypsin (0.05% w/v) and ethylenediaminetetraacetic acid (EDTA) (0.02% w/v) in PBS and centrifugation (250 g, RT, 10 min), single cell suspensions were obtained with a viability of at least 95% (Griffini et al. 1997).

Surgery

A small midline incision was made in the abdominal wall of rats under anesthesia with FFM mix (1 ml Hypnorm, 1 ml Midazolam, and 2 ml water, 0.27 ml/100 g body weight IP). A suspension containing $5 \times 10^5$ cancer cells in 500 μl PBS was injected into the portal vein with a 27-gauge needle. The portal vein was closed by treatment of the incision with an aqueous solution of thrombin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service; Amsterdam, The Netherlands) using a cotton bud. After 3 weeks, on average 10 metastases had developed per liver, particularly at the surface of liver lobes, with diameters ranging between 0.5 and 3.0 mm. The rats were sacrificed with an overdose of sodium pentobarbital and the livers were removed immediately and cut into small cubic pieces (5 mm thick), which were frozen in liquid nitrogen and stored at -80°C until used. Some metastases were isolated from liver parenchyma and separately frozen in liquid nitrogen. From one control animal, kidney, and duodenum were removed and frozen in liquid nitrogen. Serial sections (8 μm thick) were cut at a cabinet temperature of -25°C using a motor-driven cryostat (Bright; Huntingdon, UK).

Gelatin zymography

MMPs in tissue extracts of liver metastases were determined by modification of the sodium dodecyl sulfate (SDS)-poly-acrylamide gel electrophoresis (PAGE) procedure (Heussen and Dowdle 1980; Creemers et al. 1998). Tissues were ultrasonically homogenized: 15.0 mg wet tissue in 0.2 ml extraction buffer [1% (w/v) Triton X-100, 50 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 10 mM CaCl$_2$] (Forsyth et al. 1998). After an overnight incubation at 4°C, the samples were centrifuged for 10 min at 9700 g. Supernatants (15 μl) were diluted 1:4 with sample buffer (0.1 M Tris-HCl, pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, 0.005% (w/v)
bromophenol blue, 10 mM EDTA) and electrophoresed, together with molecular weight markers, on a 10% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, gels were washed for 30 min in a buffer containing 50 mM Tris-HCl (pH 7.5), 2.5% (w/v) Triton X-100, and 5 mM CaCl$_2$ and incubated overnight (16 hr) in incubation buffer at 37°C [1% (w/v) Triton X-100, 50 mM Tris-HCl, 5 mM CaCl$_2$, 0.02% (w/v) Na$_3$PO$_4$, pH 7.6]. Gels were stained with Coomassie blue and destained by acetic acid in methanol and dH$_2$O (1:3:6), both for 60 min, to visualize bands with gelatinolytic activity (Woessner 1995; Oh et al. 1999). Furthermore, DQ-gelatin (0.25 mg/ml) instead of regular gelatin was added to the polyacrylamide gel to ascertain that DQ-gelatin is a suitable substrate for MMP-2 and MMP-9. Further procedures were similar to those described above. To characterize the enzyme(s) responsible for gelatin breakdown, the following protease inhibitors were added to the incubation buffer: 20 mM EDTA (general MMP inhibitor), 10 μM CT-1166; Celltech, Klamath Falls, OR (selective inhibitor of MMP-2 and MMP-9), 20 μM pepstatin A (inhibitor of aspartate proteinases), 1 mM phenylmethanesulfonyl fluoride (PMSF; inhibitor of serine proteinases) and 50 μM trans-epoxysuccinyl-l-leucylamido (4-guanidino)-butane (E64, inhibitor of cysteine proteinases), all three obtained from Sigma, (Zwijndrecht, The Netherlands).

**Immunohistochemistry**

Cryostat sections (8 μm thick) of livers containing metastases were air-dried for 1 hr and fixed in acetone at RT for 10 min. Colon cancer cells were detected with Ulex europaeus agglutinin-1 conjugated to horseradish peroxidase (UEA-HRP; EY Laboratories, San Mateo, CA), a selective marker of colon carcinoma cells (Griffini et al. 1997). Peroxidase activity was visualized by incubation for 10 min at RT with a solution containing 20 mg 3-amino-9-ethylcarbazole (Sigma), 5 ml dimethylformamide, and 0.01% hydrogen peroxide in 50 mM sodium acetate buffer (pH 4.9). After rinsing in dH$_2$O, sections were counterstained with hematoxylin and mounted in glycerin–gelatin. MMP-2 was demonstrated with a mouse monoclonal antibody against human active and inactive MMP-2 (diluted 1:800; Neomarkers, Fremont, CA) and collagen type IV with a goat polyclonal antibody against human collagen type IV (diluted 1:500; SBA, Birmingham, AL). Crossreactivity of the anti-human antibodies with rat MMP-2 and collagen type IV, as described by the manufacturer, was tested. Incubations with the first antibodies were performed for 1 hr at RT in PBS containing 0.2% (w/v) bovine serum albumin (BSA). After rinsing in PBS for 15 min, sections were incubated
with rabbit anti-mouse and rabbit anti-goat immunoglobulins coupled to TRITC or FITC (Dako; Glostrup, Denmark), diluted 1:200, in the presence of 0.2% (w/v) BSA and 1% (v/v) normal rat serum for 1 hr at RT. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1.0 µg/ml). The specificity of the IHC procedures was determined by omitting primary antibodies in the first incubation.

In situ zymography

Gelatinolytic activity was demonstrated in unfixed cryostat sections (8 µm thick) using DQ-gelatin as a substrate (Enz-Chek; Molecular Probes, Eugene, OR). Cryostat sections of livers containing metastases, kidney, and duodenum were air-dried for 10 min. DQ-gelatin was dissolved in a concentration of 1 mg/ml in water and then 1:10 diluted in 1% (w/v) low gelling temperature agarose (Sigma) in PBS containing DAPI (1.0 µg/ml) or propidium iodide (PI; 0.5 µg/ml) to counterstain nuclei (Weber et al. 1999). The mixture (40 µl) was put on top of the sections and covered with a coverslip. After gelling of the agar at 4°C, the incubation was performed for 1 hr at RT. Fluorescence of FITC was detected with excitation at 460–500 nm and emission at 512–542 nm. DAPI was detected with excitation at 340–380 nm and emission at 425–490 nm. PI was detected with excitation at 540–580 nm and emission at 608–683 nm. Specificity of the gelatinolytic activity due to MMP activity was determined with 20 mM EDTA and 10 µM CT-1166. The effects of the same inhibitors that were used for gelatin zymography (see above), either separately or in a mixture, were also studied. Cryostat sections were preincubated for 1 hr at RT with the different inhibitors dissolved in PBS. Then incubation with DQ-gelatin was performed as described above in the presence of the inhibitors. To ascertain the enzymatic nature of the appearance of fluorescence and its specific localization patterns, collagenase-degraded DQ-gelatin and unconjugated FITC (fluorescent peptides and fluorescent molecules, respectively) were applied to the sections. Furthermore, 4% formaldehyde fixation of sections for 10 min was applied to inactivate enzymes.

In situ zymography combined with IHC

For combined localization of gelatinolytic activity and MMP-2 protein or collagen type IV in the same section, IHC was performed as described above, with a TRITC-labelled secondary antibody, followed by in situ zymography with DQ-gelatin. Nuclei were counterstained with DAPI (1.0 µg/ml). Fluorescence of FITC was detected with excitation at 460–500 nm and emission at 512–542 nm and fluorescence of TRITC was detected with excitation at 540–580
nm and emission at 608–682 nm. DAPI was detected with excitation at 340–380 nm and emission at 425–∞ nm.

Results

Gelatin zymography

Gelatin zymography performed on a series of tumor homogenates showed similar gelatinolytic patterns. The banding patterns represented proteins responsible for breakdown of gelatin. On the basis of molecular weights, the following gelatinolytic enzymes could be identified in the tumors: inactive pro-MMP-9 (92 kD), inactive pro-MMP-2 (72 kD), and active forms of MMP-2 (62 and 59 kD) (Figure 1). Addition of EDTA to the incubation medium prevented formation of lysis bands, indicating enzymes responsible for gelatin degradation were MMPs. Inhibition of the formation of bands by CT1166 showed that the bands represented MMP-2 and MMP-9 (Figure 1). Inhibitors applied to inactivate aspartate, serine, and cysteine proteinases did not affect the intensity of the lysis bands (data not shown).

Figure 1. Lane 1, molecular weight markers. Gelatin zymography of a homogenate of colon cancer metastases in rat liver (Lane 2). ProMMP-9 (92 kD), proMMP-2 (72 kD), and active MMP-2 (62 kD and 59 kD) are present. Inhibition of MMP activity with EDTA (Lane 3) and CT1166 (Lane 4) prevented lysis of gelatin, indicating that the lysis bands from the tumor homogenate are due to MMP-2 and MMP-9 activity. Gelatin zymography with DQ-gelatin as substrate showed identical lysis bands, which were affected by the inhibitors similarly to the bands obtained after gelatin lysis (Figure 1). This indicates that DQ-gelatin can serve as substrate for MMP-2 and MMP-9.

Gelatin zymography using DQ-gelatin instead of gelatin as substrate for gelatinases showed identical lysis bands, which were affected by the inhibitors similarly to the bands obtained after gelatin lysis (Figure 1).
Immunohistochemistry

Tumors can be distinguished from liver parenchyma on the basis of their morphology. Cancer cells were clustered in acini, surrounded by intratumor stroma (Figure 2). MMP-2 protein was found within intratumor stroma and at basement membranes of colon cancer cells (Figure 3). Immunohistochemistry revealed that the ECM of intratumor stroma and basement membranes contained collagen type IV (Figure 4). The localization patterns of MMP-2 and collagen type IV were largely similar, suggesting a strong spatial relationship between these molecules. As positive control, immunostaining of type IV collagen was found in kidney and duodenum at basement membranes of podocytes and endothelial cells in glomeruli, and at basement membranes of epithelial cells that are known to contain collagen type IV (data not shown).

Gelatinolytic activity detected with DQ-gelatin

When DQ-gelatin was applied to unfixed cryostat sections of rat liver containing metastases, fluorescence was localized at basement membranes of colon cancer cells and in intratumor stroma. The localization had a fibrillar appearance in intratumor stroma (Figure 5). Both EDTA and CT1166, a general MMP inhibitor and a selective MMP-2 and MMP-9 inhibitor, respectively, strongly reduced fluorescence generated by breakdown of DQ-gelatin but did not completely abolish it (Figure 6). Fixation of basement membranes with formaldehyde prevented the formation of fluorescence. Inhibitors of aspartate, serine, and cysteine proteinases did not have an effect on the generation of fluorescence. In kidney and duodenum, fluorescence was not generated due to lysis of DQ-gelatin. The specificity of the generation of fluorescence due to lysis of DQ-gelatin by gelatinolytic activity was further confirmed by the findings that incubation of sections with collagenase-digested DQ-gelatin or unconjugated FITC molecules gave a homogeneous green fluorescence in the overlay but did not result in localized fluorescence.

Combined staining of gelatinolytic activity, MMP-2 protein, and collagen type IV

Combined staining of MMP-2 protein and gelatinolytic activity in the same cryostat sections showed that red and green fluorescence (MMP-2 protein and gelatinolytic activity, respectively) were localized at the same sites (Figure 7). The green fluorescence caused by DQ-gelatin breakdown was also localized in a similar way as the red fluorescence of collagen type IV immunostaining (Figure 8). Therefore, it can be concluded that MMP-2 may be
actively involved in gelatin breakdown because MMP-2 activity is present at the site of its natural substrate.

Figure 2. Immunohistochemical detection of cancer cells (red) in colon cancer metastasis in rat liver. Metastasis is surrounded by liver parenchyma (P) and consists of cancer cells (C) in tube-like and acinar structures and intratumor stroma (S). Bar = 400 μm.

Figure 3. Immunohistochemical detection of MMP-2 protein (red) in colon cancer metastasis in rat liver. MMP-2 is present in intratumor stroma (S) and at basement membranes (arrow) surrounding cancer cells (C). Nuclei are shown in blue. Bar = 50 μm.

Figure 4. Immunohistochemical detection of collagen type IV (green) in colon cancer metastasis in rat liver. Collagen type IV is present in intratumor stroma (S) and at basement membranes (arrow) surrounding cancer cells (C). Nuclei are shown in blue. Bar = 75 μm.

Figure 5. In situ zymography of gelatinolytic activity with DQ-gelatin as substrate in colon cancer metastasis in rat liver. Fluorescence due to gelatinolytic activity (green) was found in intratumor stroma (S) of tumors and at basement membranes (arrow) surrounding cancer cells (C). Nuclei are shown in blue. Bar = 50 μm.

Figure 6. In situ zymography of gelatinolytic activity with DQ-gelatin as substrate in colon cancer metastasis in rat liver. Fluorescence due to gelatinolytic activity (green) was generated in the presence of DQ-gelatin (A). Bar = 75 μm. Formation of fluorescence is strongly reduced by EDTA (B). Nuclei are shown in red. Bar = 50 μm.
Discussion

In this study we investigated the possible involvement of MMP-2 and MMP-9 in metastasis of colon cancer in rat liver. For that purpose, we used a technique for the demonstration of gelatinolytic activity in situ.

Gelatin zymography showed that MMP-2 and MMP-9, which are the major MMPs involved in gelatin breakdown, were expressed in liver metastases of colon cancer. MMP-2 was found in the biologically relevant active form, whereas MMP-9 was found only in the inactive form. Other proteinases were not found to be responsible for gelatin breakdown, based on the
experiments with various proteinase inhibitors in both gelatin zymography and in situ zymography. Therefore, in the present study we focused on the localization of MMP-2 in all further experiments. MMP-2 protein was found in intratumor stroma and around acinar structures of colon cancer cells in rat liver containing metastases. Gelatinolytic activity was localized with an in situ zymography technique using DQ-gelatin. DQ-gelatin was originally introduced for fluorometric determination of gelatinolytic activity in vitro (Della Porta et al. 1999) and was applied in the present study in an in situ assay. The principle of fluorescence production is based on the presence of quenched FITC molecules in DQ-gelatin that are liberated and start to fluoresce on proteolytic degradation of DQ-gelatin into peptides. The water-soluble fluorescent peptides are kept at the site of their production because a gelled overlay is used. The method using DQ-gelatin enables precise localization of gelatinolytic activity. Fluorescence was found at basement membranes surrounding cancer cells and in association with ECM components in intratumor stroma, whereas fluorescence was not produced in control tissues, such as kidney and duodenum. A general MMP inhibitor (EDTA) and a selective MMP-2 and MMP-9 inhibitor (CT-1166), both of which prevented the formation of lysis bands in gelatin zymograms, strongly but not completely blocked gelatinolytic activity in situ. These differences in the effects of inhibitors in zymograms and in situ zymography may be due to differences in the accessibility of MMPs in zymograms and sections for the inhibitors. Homogenates that were used for zymography have been pretreated extensively (sonification, treatment with Triton X-100 and SDS), whereas in unfixed cryostat sections the enzymes are in their natural environment, bound to other molecules.

Inhibitors of aspartate, serine, and cysteine proteinases did not affect fluorescence formation, which is in accordance with gelatin zymography showing that MMPs were the enzymes that degraded gelatin.

Because the only active gelatin-degrading proteinase was MMP-2, as judged from gelatin zymography, IHC of MMP-2 and the DQ-gelatin method to demonstrate gelatinolytic activity were performed on the same sections. These experiments were performed because localization of MMP-2 with monoclonal antibodies does not discriminate between the active and inactive protein or MMP-2 bound to TIMP. MMP-2 protein and gelatinolytic activity were localized at the same sites, suggesting that MMP-2 is at least partly responsible for the degradation of DQ-gelatin. Collagen type IV, the natural substrate of MMP-2, also showed a similar localization as gelatinolytic activity. These findings strongly suggest that active
MMP-2 is associated with its natural substrate collagen type IV. To rule out nonspecific formation of fluorescence due to interactions between components in the tissue sections and DQ-gelatin, kidney and duodenum were used for in situ zymography. These tissues are both rich in collagen type IV and express MMP-2 and MMP-9 protein but contain little MMP-2 and MMP-9 activity (Jalalah et al. 2000; Tarlton et al. 2000). DQ-gelatin was not degraded in these tissues, which indicates that DQ-gelatin degradation by gelatinolytic activity was responsible for the appearance of fluorescence in the tumors. It was excluded that FITC peptides or FITC molecules, which in theory could have been generated at other sites, have affinity for collagen by the finding that collagenase-degraded DQ-gelatin or FITC molecules did not colocalize with collagen type IV in liver metastases, kidney, and duodenum. Instead, a homogeneous fluorescence all over the section was observed. Moreover, blocking enzyme activity by formaldehyde fixation of the sections abolished the generation of fluorescence. Therefore, it can be concluded that the appearance of fluorescence from DQ-gelatin in the in situ zymography assays of colon cancer metastases is most likely due to activity of MMP-2. This conclusion was confirmed by the findings that, in both gelatin zymography and in situ zymography, gelatinolytic activity was not affected by inhibitors of proteases other than MMPs and the appearance of a lysis band due to active MMP-2 in gelatin zymography when DQ-gelatin was used as substrate. Because of the absence of a specific inhibitor of MMP-9 only, it cannot be completely excluded that MMP-9 also plays a role in the degradation of DQ-gelatin. However, this seems unlikely because no active MMP-9 was found with gelatin zymography.

Similar localization patterns of MMP-2 and collagen type IV can be explained on the basis of the molecular structure of MMP-2. MMP-2 contains an extra domain consisting of three contiguous copies of the fibronectin-derived type II homology unit (Collier et al. 1988, 1992; Massova et al. 1998; Nagase and Woessner 1999; Briknarova et al. 2001). This fibronectin-like domain is situated directly at the amino-terminal side of the zinc-binding site inside the catalytic domain (Steffensen et al. 1995; Massova et al. 1998) and accounts for the gelatin-binding properties of these MMPs. It also provides the capacity to bind to collagen type IV. Although other parts of the catalytic domain are responsible for its proteolytic activity, Murphy et al. (1994) found that this activity is largely dependent on the fibronectin-like domain. Therefore, gelatinases present in the ECM and at basement membranes of colon cancer cells may bind via their fibronectin-like domain to collagen type IV. The fibronectin-like domain possesses at least two collagen-binding sites that can be simultaneously occupied.
by two collagen molecules (Steffensen et al. 1995). This means that MMP-2 bound to collagen type IV can simultaneously bind and degrade DQ-gelatin.

In conclusion, it has been shown that gelatinolytic activity, as detected with a modified in situ zymography technique for unfixed cryostat sections, is present in colon cancer metastases in rat liver. On the basis of the findings obtained with zymography and IHC, we concluded that gelatinolytic activity was due to active MMP-2. Whether MMP-2 plays a decisive role in tumor development or is involved only in remodeling of ECM remains to be established in further experiments.

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