MMP-9-hemopexin domain hampers adhesion and migration of colorectal cancer cells

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Abstract. Matrix metalloproteinases (MMPs), in particular MMP-2 and MMP-9, are involved in colon cancer progression and metastasis due to their ability to degrade extracellular matrix (ECM) components. In previous studies we described the MMP-9 hemopexin like domain (MMP-9-PEX) as an MMP-9 antagonist. In the present study it was examined whether recombinant MMP-9-PEX has an inhibitory effect on migration and adhesion of colorectal carcinoma cells. Furthermore, we searched for MMP-9 substrate binding sites within the MMP-9-PEX by surface plasmon resonance. Migration of SW620 and LS174 cells was investigated in a modified Boyden chamber assay. In the presence of 0.2 μg/ml MMP-9-PEX migration of SW620 was decreased by 34%, while addition of 0.4 μg/ml diminished migration by 56%. Migration of LS174 cells was not affected by MMP-9-PEX. Adhesion studies were performed on 96-well plates coated with gelatin, collagen type I, and laminin, respectively. In the presence of MMP-9-PEX, adhesion of SW620 cells to these coating substrates was significantly inhibited. Surface plasmon resonance studies revealed binding of collagen type I and IV, elastin, and fibrinogen to proMMP-9 as well as to MMP-9-PEX. However, equilibrium constants (K_d) indicated a higher affinity of proMMP-9 to the matrix proteins. This could indicate that there is more than one binding site for matrix components within the entire proMMP-9 molecule. Since migration and adhesion of metastatic colorectal carcinoma cells were reduced by MMP-9-PEX, this recombinant MMP-9 antagonist might be of therapeutical interest.

Introduction

Colorectal cancer contributes to a high mortality rate in Western society (1), where metastases are the main cause of death. The metastatic process includes migration crossing the basement membrane, intravasation into vessels, transport within the circulation, adhesion to endothelial cells, extravasation through the vessel wall, as well as sedation and proliferation in response to organ-specific factors at new sites (2,3).

Degradation of extracellular matrix (ECM) respresents a hallmark for the development of secondary tumors due to metastasis (4). Cell adhesion and tumor cell migration depend on controlled ECM degradation (5), in which matrix metalloproteinases (MMP) play an important role (6). MMPs represent a family of zinc metallo-endopeptidases. Their activities are precisely regulated at the level of transcription, activation of the latent enzymes, interaction with specific ECM components, and inhibition by specific endogenous inhibitors. Cancer cells are known to recruit stromal MMP production for invasion and metastasis (7,8).

Gelatinases (MMP-2 and MMP-9) are involved in colorectal cancer progression in animal models as well as in patients (9). MMP-2 and -9 differ from other MMPs by containing a fibronectin type II domain inside the catalytic domain (10). They are able to degrade basement membranes and components of the extracellular matrix such as type IV, V, VII, X, XI, and XIV collagens, gelatin, elastin, aggrecan, proteoglycan core protein, myelin basic protein, fibronectin, fibrillin-1, as well as precursors of TNF-α, IL-1ß, and others (11). Especially MMP-9 expression correlated with local tumor growth (12), invasion, and intrahepatic metastasis, e.g. of hepatocellular carcinomas (13). On the other hand inhibition of MMP-9 expression blocked metastasis in a rat sarcoma model system (14). Furthermore, up-regulation of MMP-9 has been used in a portal blood assay to predict liver metastasis of colorectal cancer (15). Enhanced MMP-9 expression in primary colon tumors was associated with liver metastasis (16,17) and MMP-9 expression was increased in biopsy samples from patients with colon cancer in comparison to healthy bowel tissue of the same patient (8).

MMP-9 differs from MMP-2 by a unique linker sequence of unknown function connecting the active site and the hemopexin domain (18). Substrate specificity of MMP-9 is tightly connected to the hemopexin domain which consists of four units of four-stranded antiparallel ß sheet stabilized on its 4-fold axis by a cation (Zn^{2+}). This domain constitutes a 4-bladed ß-propeller structure in which the blades are scarcely twisted (19). One remarkable property of MMP-9-PEX is the lack of stabilizing elements, present in all other related

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hemopexin-like structures. MMP-9-PEX is the first hemopexin structure determined in a dimeric state (20). The loss of few amino acid residues at the carboxy terminus inhibited substrate binding and inhibition by TIMP-1. Our previous study showed that MMP-9 activity is specifically reduced by its own hemopexin-like domain expressed in *E. coli* (21).

There is increasing evidence that a specific inhibition of MMP-9 activity might prevent metastasis. However, the exact molecular mechanisms of MMP-9 involvement in the metastatic process remain unknown. Identification of ECM components involved in this process and the investigation of highly specific MMP-9 antagonists may by useful for the development of new therapeutic approaches to lower the metastatic potential of colon carcinoma cells.

The aim of the present study was to demonstrate that MMP-9-PEX influences the migration of colon cancer cells. Furthermore, the influence of MMP-9-PEX on adhesion to ECM components was investigated. To specifically address the ability of MMP-9-PEX to bind ECM components, binding profiles of MMP-9-PEX for collagen type I/IV, gelatin, elastin, and fibrinogen were established using surface plasmon resonance (SPR).

Materials and methods

Materials. Culture reagents were obtained from Sigma-Aldrich (Steinheim, Germany), Gibco (Eggenstein, Germany), Sarstedt (Berlin, Germany) or PAA (Cölbe, Germany). All chemicals were purchased from Sigma-Aldrich (Munich, Germany), and GE Healthcare (Freiburg, Germany).

ProMMP-9 and MMP-9-PEX analysis. For antibody-based detection, buffer samples were separated by SDS-PAGE utilizing separating gels of 10% and 12.5% polyacrylamide for proMMP-9 and MMP-9-PEX, respectively and stacking gels of 3% polyacrylamide. Electrophoresis was performed at 40 mA. Silver staining of polyacrylamide gels and Western blotting were performed according to a method described previously (22). Briefly, the proteins were transferred to a PVDF membrane at 2 mA/cm^2. Blots were blocked with TBS-N (pH 7.6) containing 10% BSA, 20 mM Tris, 137 mM NaCl, and 0.1% Nonidet P40, and were washed and incubated with antibodies against the C terminus of murine MMP-9 (AF 909, R&D Systems, dilution 1:1000). Signals were visualized by enhanced chemiluminescence according to the manufacturer's instructions (ECL detection reagents, Amersham Biosciences).

Expression and purification of MMP-9-PEX. BL21 *E. coli* cells were transformed with the pGEX-5X-1-PEX plasmid and the MMP-9-PEX-GST fusion protein was expressed according to a method described by Roeb *et al* (21). Collected fractions were analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and the MMP-9-PEX-GST fusion protein was identified by immunoblot analysis as described above.

ProMMP-9 purification. Recombinant proMMP-9 was produced as described previously (23). Full-length mouse proMMP-9 was subcloned into a high-expression replication-deficient adenovirus type 5 with transcription under the control of the cytomegalovirus promotor. The ~2.9-kbp fragments of pRc/CMV-mGelB was integrated into the vector pΔE1sp1A-CMV-EGFP, depleted for EGFP. The integrity of all cloning boundaries was verified by sequencing and the entire insert was shuttled to the adenovirus plasmid (24). The adenovirus was selected, purified and expressed as previously described (23). Recombinant proMMP-9 was generated by infecting human hepatoma cells (HepG2), cultivated as described before (25), using a virus concentration of 2x10^8 pfu/ml in DMEM-F12 containing 5% (v/v) heat-inactivated fetal calf serum (FCS), 100 mg/l streptomycin, and 60 mg/l penicillin for 16 h. Cells were cultured for 24 h with renewed medium, and thereafter grown under serum-free conditions for 48 h before harvest. The harvested HepG2 conditioned media were sterile-filtered, and dialyzed against 'native buffer' (NB) containing 100 mM Tris/HCl, pH 7.5; 50 mM NaCl; 1 mM $CaCl_2$; 0.02% (w/v) PEG6000. The recombinant proMMP-9 was isolated from the pre-purified samples in a one-step affinity chromatography utilizing gelatin-sepharose. Protein purity and integrity was checked by SDS-PAGE, subsequent silver staining, and Western blot analysis.

Colon cancer cells and cell treatment. Human colon cancer cell lines SW620 (ATCC, CCL-227) and LS174 (ATCC, CL-188) were employed for this study. SW620 and LS174 cells were maintained in RPMI-1640 supplemented with 10% FCS, streptomycin (10 mg/l), and penicillin (10 U/l). Cells were grown at 5% CO_2 and 37˚C in a water saturated atmosphere. One day prior to experiments cells were seeded to provide a final cell density of 60-70% confluence.

Boyden chamber experiments. Transwell 8-μm pore membrane inserts (18-mm standard PCTE filters, Neuro Probe, Gaithersburg, USA) were activated for 20 min at 50˚C with 0.5% acetic acid. The activated membranes were coated with 5 mg/l gelatin solution for 1 h at 100˚C (26). After drying (100˚C, 1 h on Whatman paper) the inserts were placed in a blind well chemotaxis chamber (Neuro Probe). The lower compartment was filled with 50 μg/ml collagen type I (from human placenta) containing fibroblast conditioned medium according to the method of Wach *et al* (27), which was used as chemoattractant. Prior to seeding cells were detached by trypsin treatment, rinsed with FCS-free medium and diluted to 2.5x10^5 cells/ml. Cell suspension (800 μl) with PEX or BSA as reference protein or without further supplements was added to the upper chamber. Chambers were placed in a humified tissue incubator, containing 5% CO_2 for 16 h at 37˚C. Cells on the upper surface of the transwell inserts were removed using a cotton swab and those on the lower surface of the membranes were fixed with 10% methanol and stained with hematoxylin and eosin. Membranes were rinsed with deionized water, dried, and examined using light microscopy. The number of migrated cells in five optical fields (magnification x400) was averaged.

Attachment assay. Attachment assays were performed in 96-well plates. Gelatin-, collagen type I- or laminin-coated plates were used. Plates were coated with 0.4% gelatin in deionized water for 2 h at 37˚C. For collagen coating, wells

were incubated with collagen type I solution [50 μg/ml phosphate-buffered saline (PBS), prepared according to the manufacturer's instructions] for 2 h at 0˚C, then the solution was removed and the plates were dried at 0˚C. Fifteen μg laminin per ml PBS was used for laminin coating. Wells were filled with laminin solution and incubated at 4˚C overnight. Colon carcinoma cells were harvested by trypsin incubation and resuspended in medium. Fifty-thousand cells per well were used for the assay. Standard curves for every plate were generated by seeding 12500, 25000, 50000 and 100000 cells per well in duplicate respectively. Plates were incubated overnight in a humified tissue incubator with 5% CO_2 and at 37˚C. After removal of the supernatant, the attached cells were fixed for 10 min using 1% glutaraldehyde and stained with 0.1% crystal violet solution for 25 min. Wells were rinsed with deionized water and treated with 0.1% Triton X-100 solution. Absorption of the resolved crystal violet was measured photometrically at 540 nm.

Surface plasmon resonance studies. Gelatin, collagen type I, and type IV was covalently immobilized to a carboxymethyl dextran matrix (Fisons, Loughborough, UK) at 50 μg/ml respectively for 2 min in 10 mM sodium acetate buffer, pH 3.9 as recommended by the manufacturer. Elastin was immobilized stepwise, starting with 10 μl elastin stock solution 1 (200 mg/ml PBS, pH 6.0) + 90 μl phosphate-buffered saline, pH 6.0 followed by the addition of 10 μl 10 mM acetic acid. Two-hundred μl elastin stock solution 1 was injected in a second step. Finally, 200 μl elastin stock solution 2 (250 mg/ml aqua dest.) was added. Elastin was immobilized to the membrane for 5 min. Fibrinogen [10 μl fibrinogen stock solution (100 mg/ml PBS, pH 6.0) + 90 μl 10 mM NaAc pH 4.5] was covalently immobilized to the carboxymethyl dextran for 5 min. Binding experiments were performed at a controlled temperature (15˚C) with different concentrations of proMMP-9 and MMP-9-PEX using the IASYSTEM (Fisons) optical biosensor. Association was monitored for at least 2 min. The sample was replaced by PBS containing 0.1% Triton X-100 solution (PBS-T), pH 7.4 and dissociation was monitored accordingly before the cuvette was regenerated with 10 mM HCl and equilibrated again. Association and dissociation affinograms were analyzed by non-linear regression with FASTfit software (Fisons), which uses the Marquardt-Levenburg algorithm for iterative data fitting.

Statistical analysis. Statistical significance ($p<0.05$) between controls and samples (n=8-145) was determined by unpaired t-tests postulating different variances (Welch's t-test) and SED (standard error of differences) was calculated as SD (standard deviation) of the sample distribution (sample mean).

Results

Purification and characterization of MMP-9-PEX. Recombinant GST-MMP-9-PEX fusion protein was expressed in *E. coli* (BL21). Bacterial lysates were subjected to SDS-PAGE and subsequent Western blotting. Expression revealed a GST-MMP-9-PEX fusion protein corresponding to a molecular mass of 52 kDa detected by anti-murine MMP-9 antibodies. Recombinant GST-MMP-9-PEX digested by factor Xa and

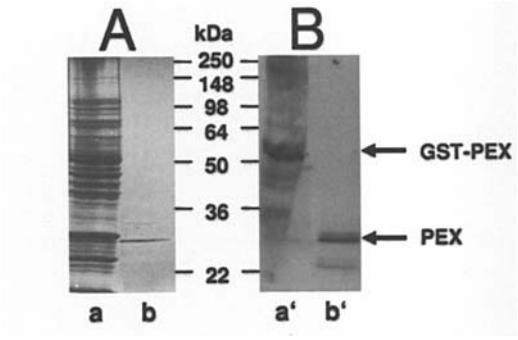

Figure 1. Purification and characterization of recombinant MMP-9 PEX. A, coomassie-stained SDS-PAGE of bacterial lysates (a) and of the purified MMP-9-PEX (b); B, anti-murine MMP-9 Western blot analysis of bacterial lysates (a') and of the purified MMP-9-PEX (b').

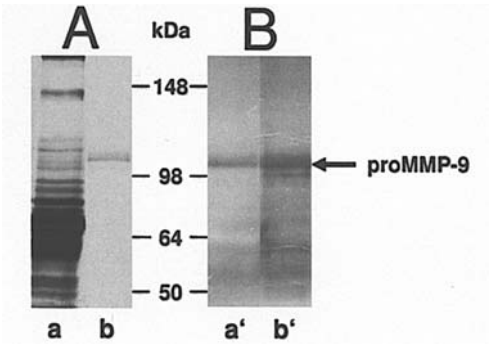

Figure 2. Purification and characterization of proMMP-9. A, silver-stained SDS-PAGE of cell culture supernatant of transfected cells (a) and of the purified proMMP-9 (b); B, anti-murine MMP-9 Western blot analysis of cell culture supernatant of transfected cells (a') and of the purified proMMP-9 (b').

the cleavage products were isolated by gel filtration in a second column purification step. After final purification a 25-kDa protein was detected by coomassie-stained SDS-PAGE (Fig. 1A). The identity of MMP-9-PEX was proved by Western blot analysis (Fig. 1B) with specific anti-murine MMP-9 antibodies.

Purification and characterization of proMMP-9. Recombinant proMMP-9 was enriched and purified from conditioned cell culture medium by affinity chromatography using gelatin-sepharose. Fifty to two-hundred μg (of ~80-90% purity) of proMMP-9 were extracted from 200 ml cell culture medium. Conditioned cell culture media and purified material were analysed by silver-stained SDS-PAGE (Fig. 2A) and Western blotting (Fig. 2B).

Migration of colon carcinoma cells in the presence of MMP-9-PEX. To investigate whether MMP-9-PEX influences the migration of colon carcinoma cells, we examined the motility of SW620 and LS174 cells in Boyden chamber experiments. Type I collagen containing fibroblast conditioned medium was used as chemoattractant. After incubation for 16 h, migrated cells on the underside of the gelatin-coated membrane were counted. Experiments were performed in triplicate. The median cell number of five optic fields per membrane was determined. Data were normalized by setting the number of

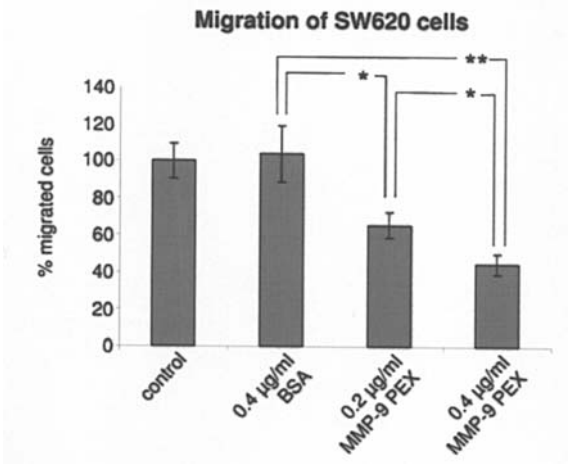

Figure 3. Effect of MMP-9-PEX addition on migration of SW620 cells. Relative migration of SW620 cells was measured in Boyden chambers without MMP-9-PEX, with addition of 0.4 μg/ml BSA as reference protein and with 0.2 and 0.4 μg/ml MMP-9-PEX. Cells on the lower surface of the membrane were counted after staining with hematoxylin and eosin and the cell number of five optical fields was averaged. y-axis, number of cells in %. Each bar represents the mean ± SED of three chambers. *p<0.05; **p<0.001.

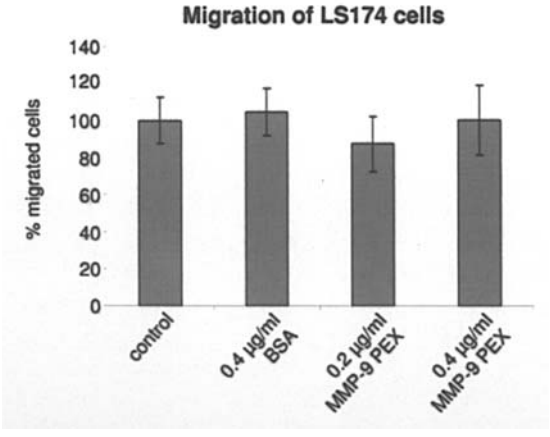

Figure 4. Effect of MMP-9-PEX addition on migration of LS174 cells. Relative migration of LS174 cells was measured in Boyden chambers without MMP-9-PEX, with addition of 0.4 μg/ml BSA as reference protein and with 0.2 and 0.4 μg/ml MMP-9-PEX. Cells on the lower surface of the membrane were counted after staining with hematoxylin and eosin and the cell number of five optical fields was averaged. y-axis, number of cells in %. Each bar represents the mean ± SED of three chambers.

migrated control cells (no MMP-9-PEX or BSA) to 100%. Data are presented as mean ± SED. Metastasis-derived SW620 cells showed a dose-dependent decrease in migration through the membrane. The addition of 0.2 μg/ml MMP-9-PEX led to a 34.4±6.8% reduction (p=0.034) and 0.4 μg/ml to a 55.5±5.4% reduction of migration (p=0.003, Fig. 3). In contrast, migration of primary tumor-derived LS174 cells remained unchanged after addition of MMP-9-PEX in different concentrations (Fig. 4).

Adhesion of SW620 colon carcinoma cells in the presence of MMP-9-PEX. Since adhesion of tumor cells to endothelial cells is mediated by ECM proteins, we investigated the adhesion of SW620 cells to gelatin-, collagen type I-, and laminin-

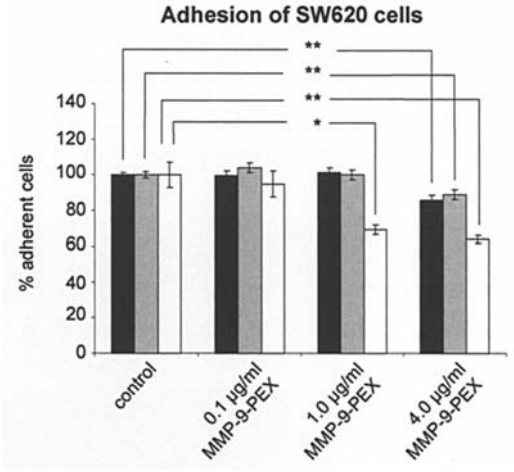

Figure 5. MMP-9-PEX addition reduces adhesion of SW620 cells on gelatin-, collagen type I- and laminin-coated surfaces. Relative adhesion of SW620 cells was measured in coated 96-well plates without MMP-9-PEX and with 0.1, 1.0 and 4.0 μg/ml MMP-9-PEX. Adherent cells were stained with crystal violet. After washing and treatment with 0.1% Triton X-100 solution resolved crystal violet was measured photometrically at 540 nm. Relative adhesion was calculated using a standard curve. y-axis, relative cell adhesion in %. Each bar represents the mean ± SED of eight values. Collagen type I, black bars; laminin, grey bars; gelatin, white bars; *p<0.05; **p<0.001.

coated surfaces either in the presence or absence of MMP-9-PEX. Cells were seeded to coated 96-well plates and incubated for 16 h with 0.1, 1.0, and 4.0 μg/ml MMP-9-PEX or 4 μg/ml BSA as control. Relative adherence was measured by setting the number of adherent cells without MMP-9-PEX to 100%. Data were calculated as mean ± SED of 8 wells respectively. The adhesion of SW620 cells on collagen type I was reduced by 14.1±2.6% while adhesion to laminin decreased by 11.2±2.7% in the presence of MMP-9-PEX (4 μg/ml). Adhesion to gelatin was also affected. The number of adherent cells was reduced by 30.6±2.8% (1 μg/ml MMP-9-PEX) and by 36.2±2.2% (4 μg/ml) (Fig. 5). Thus a significant reduction of SW620 cell adhesion by MMP-9-PEX was observed for all surfaces examined.

Binding of MMP-9-PEX to gelatin, collagen type I and IV, elastin and fibrinogen. In the present study we measured the affinity of MMP-9-PEX to physiological ECM substrates (collagen type I and IV, elastin, fibrinogen) in comparison to the entire enzyme using representative surface plasmon resonance experiments are shown in Fig. 6 as described in Materials and methods. Immobilisation of other ECM components, such as laminin, decorin, aggrecan, and fibronectin, was not effective and due to this SPR was abrogated. The association (k_{on}) and dissociation (k_{off}) rate constants of proMMP-9 and MMP-9-PEX for different substrates were determined and the equilibrium constant (K_d) was calculated respectively (Tables I and II).

MMP-9-PEX exhibited binding to gelatin, collagen type I and IV, elastin, and fibrinogen. The highest affinities were found for gelatin (K_d = 295 nM), collagen type I (K_d = 444 nM), and collagen type IV (K_d = 655 nM). Weaker affinities were observed for elastin (K_d = 1.1 μM) and for fibrinogen (K_d = 1.26 μM) (Table I). In contrast to that proMMP-9 showed very high affinities for collagen type IV (K_d = 30.8 pM) and

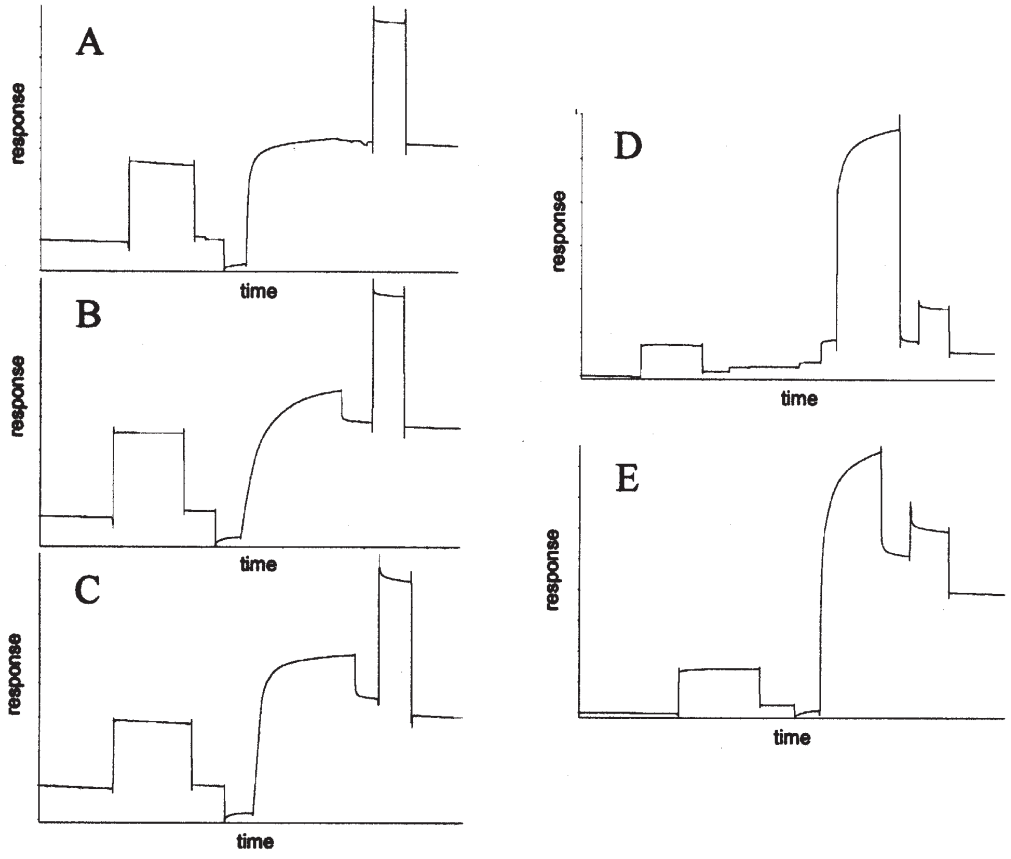

Figure 6. Immobilisation of ECM proteins. Covalent binding profiles of ECM components to a carboxymethyl dextran matrix. Immobilisation of: A, gelatin; B, collagen type I; C, collagen type IV; D, elastin; E, fibrinogen.

Table I. Kinetic (k_{on}, association; k_{off}, dissociation) and equibrilium (K_d, dissociation) constants for the binding of MMP-9-PEX to ECM proteins.

ECM protein	k_{on} $M^{-1}s^{-1}$	k_{off} s^{-1}	K_d M
Gelatin	$5.62x10^4 \pm 2.65x10^3$	$3.71x10^{-3} \pm 2.29x10^{-4}$	$2.95x10^{-7} \pm 3.84x10^{-8}$
Collagen type I	$4.22x10^4 \pm 1.20x10^3$	$5.33x10^{-3} \pm 2.88x10^{-4}$	$4.44x10^{-7} \pm 6.21x10^{-8}$
Collagen type IV	$7.45x10^3 \pm 5.61x10^2$	$3.93x10^{-3} \pm 2.59x10^{-4}$	$6.55x10^{-7} \pm 1.18x10^{-7}$
Elastin	$8.61x10^2 \pm 4.31x10^1$	$9.50x10^{-4} \pm 3.24x10^{-5}$	$1.10x10^{-6} \pm 9.29x10^{-8}$
Fibrinogen	$6.51x10^3 \pm 3.75x10^2$	$4.36x10^{-3} \pm 1.18x10^{-4}$	$1.26x10^{-6} \pm 1.23x10^{-7}$

Table II. Kinetic (k_{on}, association; k_{off}, dissociation) and equibrilium (K_d, dissociation) constants for the binding of the proMMP-9 to ECM proteins.

EECM protein	k_{on} $M^{-1}s^{-1}$	k_{off} s^{-1}	K_d M
Gelatin	$7.18x10^5 \pm 5.47x10^4$	$1.89x10^{-2} \pm 9.77x10^{-4}$	$3.29x10^{-8} \pm 4.20x10^{-9}$
Collagen type I	$5.74x10^9 \pm 4.07x10^8$	$4.66x10^{-2} \pm 3.28x10^{-3}$	$4.28x10^{-8} \pm 6.06x10^{-9}$
Collagen type IV	$1.14x10^9 \pm 1.00x10^8$	$6.85x10^{-3} \pm 5.34x10^{-4}$	$3.08x10^{-11} \pm 5.12x10^{-12}$
Elastin	$9.36x10^7 \pm 1.19x10^7$	$5.02x10^{-3} \pm 4.03x10^{-4}$	$6.28x10^{-11} \pm 1.30x10^{-11}$
Fibrinogen	$4.79x10^7 \pm 4.70x10^6$	$1.13x10^{-2} \pm 1.05x10^{-3}$	$1.51x10^{-9} \pm 2.88x10^{-10}$

Table III. Interaction between MMP-9-PEX and ECM proteins.

ECM protein	Adhesion	SPR
Gelatin	+++	+++
Collagen type I	++	+++
Collagen type IV	ND[a]	++
Laminin	+	ND[a]
Elastin	ND[a]	+
Fibrinogen	ND[a]	+

[a]ND, not detected.

elastin (K_d = 62.8 pM). The corresponding binding for MMP-9-PEX was ~10^4 times weaker. The affinity of proMMP-9 to fibrinogen (K_d = 1.51 nM) was found to be ~10^3 times higher than the corresponding MMP-9-PEX affinity. Concerning gelatin and collagen type I equilibrium constants for complex formation with proMMP-9 (K_d = 32.9 nM, K_d = 42.8 nM respectively) were approximately 10 times higher than the values for MMP-9-PEX. In summary, we observed a similar affinity for MMP-9-PEX to gelatin and collagen type I.

Discussion

Tumor cell traversal of basement membrane barriers is the result of the acquisition of an invasive phenotype: tumor cells acquired the ability to attach to the ECM, to degrade matrix components, and then migrate through these matrix defects. Metastasis is therefore a multistep process involving numerous tumor-cell host interaction. These interactions are defined by the invasive phenotype that can be separated into three steps: attachment, local proteolysis, and migration (7,28-30). Hence local ECM proteolysis is essential for tumor invasion and depends strongly on the activity of MMPs. Commonly, MMPs play a major role in cell motility in different types of tissue (31). The role of MMPs due to cell invasion and metastasis has been the subject of a variety of studies in the last year (32-36). We have recently demonstrated a positive correlation between MMP-9 expression and development of colorectal carcinomas (8). Others found that active MMP-9 was more strongly expressed in colon carcinomas from patients with liver metastases than from patients without metastasis (16).

In the last decade several synthetic inhibitors of MMPs were developed and went rapidly into clinical trials. In most of these trials the inhibitors were tested in patients with end-stage disease without the complete knowledge of the complex mechanism of action of MMPs in cancer (37). These trials failed to reach their end points of increased survival. Therefore critical assessments of the criteria for successful therapeutic MMP-inhibition were provided (36,38-40). In conclusion these results suggest that a successful anticancer inhibitor must possess MMP selectivity against those MMP subtypes whose involvement is critical (41). MMP-9 could be a key therapeutic target in patients with colorectal cancer (42). Due to this MMP-9 inhibition might be beneficial in colorectal tumor progression. Recently we characterized the MMP-9 C-terminal hemopexin domain and demonstrated its MMP-9 antagonistic activity (21). Furthermore we showed that migration of malignant melanoma cells overexpressing MMP-9 was reduced in the presence of MMP-9-PEX. Therefore, MMP-9-PEX might have an impact on the metastatic potential of tumor cells.

In the present study we examined the effect of MMP-9-PEX on adhesion and migration of colon cancer cell lines SW620 and LS174, derived from metastases and primary tumor material respectively. Two of the three central steps of the metastatic cascade, cell adhesion and cell migration, were inhibited by MMP-9-PEX in highly metastatic SW620 cells. Interestingly, the motility of primary tumor-derived LS174 cells remained unaffected by MMP-9-PEX. In the presence of MMP-9-PEX we further observed a decrease in cell adhesion to matrix proteins (collagen type I, gelatin, and laminin). The antagonistic effects of MMP-9-PEX on migration and adhesion of colon carcinoma cells might lower their invasive potential.

The gelatin binding property of MMP-9-PEX has already been described (21). We now focused on interactions between MMP-9-PEX and physiological ECM components, such as collagen type I. The interactions of MMP-9-PEX and ECM proteins were summarized in Table III. We performed surface plasmon resonance (SPR) studies with selected matrix proteins according to Björklund and Koivunnen (43). In addition to collagen type I and IV, elastin, and fibrinogen the binding properties of MMP-9-PEX to gelatin was tested and thus the high affinity interactions between MMP-9-PEX and gelatin, published earlier, were confirmed (21).

A comparable binding capacity could be identified between MMP-9-PEX and collagen type I (K_d = 444 nM). Interestingly MMP-9 is unable to cleave soluble or fibrillar collagen type I (44). The binding of MMP-9 to collagen type I has already been described (10). It was postulated that only the N-terminal portion of the molecule should be responsible for gelatin binding. In a recent study, however, a unique collagen-binding domain (CBD) consisting of three fibronectin type II-like modules was identified for MMP-9 and MMP-2 (45). This CBD was able to bind a series of collagen types including collagen type I/IV. Recombinant CBDs of MMP-2 and MMP-9 were shown to inhibit gelatinolytic activity of both enzymes and vice versa. Interestingly inactive mutants of MMP-2 and MMP-9 had a stronger inhibitory effect on gelatinolysis indicating unresolved interactions between the CBD and other parts of the enzyme. This observation is consistent with previous results demonstrating that the hemopexin-like domain of MMP-2 is involved in collagenolysis (46). Due to our results MMP-9-PEX is involved in the binding of collagen type I and gelatin. Digestion of collagen type I, the initial step of ECM degradation (47), is promoted by trypsin, which is co-expressed with MMPs in colorectal carcinomas (36). Furthermore trypsin activates a number of pro-collagenases, including proMMP-2 and proMMP-9. In this context the close localization of MMP-9 and collagen type I might be associated with the colorectal cancer promoting effect of trypsin, e.g. degradation of collagen type I and activation of MMP-9. Our results imply that these interactions may be reduced in the presence of MMP-9-PEX.

Binding capacities of MMP-9-PEX for collagen type IV, elastin, and fibrinogen, which are all MMP-9 substrates, have also been identified (43). The equilibrium constants between MMP-9-PEX and the substrates collagen IV, elastin and fibrinogen were lower than for gelatin and collagen type I. For the entire enzyme, however, we observed high affinities for collagen type IV, elastin, and fibrinogen. Contrary to our results Olson *et al* found low affinities of proMMP-9 for collagen type IV (K_d = 2.15 μM) and the purified α2(IV) chain of collagen type IV (K_d ~45 nM). Others also described a degradation of laminin (48), fibrinogen (49), and elastin (50) by MMP-9, but the kinetics of the interactions or binding affinities for the substrates were not presented. It has been proposed recently that MMP-9 interacts with several cell surface components namely the surface-associated α2(IV) chain of collagen type IV, hyaluron receptor CD44, RECK (reversion-inducing-cystein-rich protein with Kazal motifs), and LRP (low-density lipoprotein receptor-related protein) (51). This type of interaction positively regulates enzymatic activation and activity (51). A major dilemma in the understanding of MMP-9 function is how the released protease is targeted to the right location and how its activity is controlled within the extracellular space. The interaction of MMP-9-PEX with physiological ECM components, such as collagen type I and IV, elastin, and fibrinogen, may play a critical role in localization of the MMP-9 to focus and restrict its activity to the pericellular environment.

Our results demonstrate that migration of metastatic colorectal cancer cells is reduced in the presence of MMP-9-PEX. Furthermore the adhesion of these cells on matrix components is inhibited by MMP-9-PEX. This indicates that the interactions of MMP-9 with gelatin, collagen type I, and laminin were reduced by MMP-9-PEX. The underlying molecular mechanisms have to be clarified in further studies. From our data we conclude that the hemopexin-like domain of MMP-9 contains exosides for collagen type I and for gelatin. Furthermore, our data show that MMP-9-PEX has additional binding sites for collagen type IV, elastin, and fibrinogen.

We provide new aspects of MMP-9 binding properties and show that MMP-9-PEX might be a promising approach for antimetastatic therapy. The impact of MMP-9-PEX on colorectal cancer progression, however, needs further evaluation.

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