Cell-cell interactions in the gastrointestinal tumour-microenvironment

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Chapter 7

Endothelium specific matrilysin (MMP-7) expression in human cancers

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Abstract

Over-expression of matrilysin (MMP-7) is predominantly associated with epithelial (pre)malignant cells. In the present study MMP-7 expression is also found in endothelial cells in various human cancer types. Endothelial MMP-7 was associated with CD34 and/or CD105 expression. These immunohistochemical data were confirmed by RT-PCR on VEGF-stimulated endothelial cells. In addition, MMP-7 was also identified in sprouting endothelial cells *in vitro*. The potential clinical relevance of endothelial MMP-7 was assessed for cervical cancer patients by evaluating the association with overall survival. In contrast to MMP-7 in malignant epithelial cells, MMP-7 expression in endothelial cells showed a significant association with poor survival (LR 5.12, P=0.02, n=30). Our data suggest that MMP-7 is involved in tumour angiogenesis, thereby contributing to malignant growth and hence associated with decreased survival.

Introduction

Matrilysin (matrix metalloproteinase 7, MMP-7) has long been regarded as the MMP exclusively produced by epithelial cells. Up-regulation of epithelial MMP-7 in early stage tumours is a consequence of mutations in the Wnt-signaling pathway[1]. In later tumour stages, hypoxia contributes to the induction of MMP-7 expression[2]. Only few non-epithelial cell types have been described to express MMP-7 *in vivo*. The first indication for MMP-7 production by endothelial cells in tumour tissue came from Nagashima et al.[3], but this important finding has never been confirmed. We evaluated the presence and the role of MMP-7 in endothelial cells in cancer. First, we investigated MMP-7 expression in endothelial cells within various tumour types in relation to co-expression with pan-endothelial marker CD34 and proliferating endothelium associated marker CD105[4]. Secondly, using real time RT-PCR, we determined whether VEGF, the classical initiator of angiogenesis, was able to induce MMP-7 expression in sprouting endothelial cells *in vitro*. In addition, we determined the effect of an MMP inhibitor on sprouting endothelial cells, in which MMP-7, either directly or indirectly, could be involved. Finally, to evaluate a potential clinical implication, we assessed the association between MMP-7 expression in tumour endothelial cells and survival of cervical cancer patients.

Experimental procedures

Patient material

A total of 254 patients with untreated primary cervical carcinoma underwent a radical hysterectomy type III with lymphadenectomy between 1985 and 1995. Tissue samples were fixed in 10% formalin and embedded in paraffin. Samples from 30 patients were included in this study: Mean age 45 years (range 29-72); FIGO stage: (20), IIA (8), IIB/IIIB (2); lymph node metastases: No (16), Yes (14);distant metastases: No (13), Yes (17); tumour size: <40 mm (16), ≥40 mm (12); infiltration depth <15 mm (15), ≥15 mm (9); vascular space involvement: No (10), Yes (20); parametrial invasion: No (20), Yes (10); HPV status: 16/18 (19), other types (6); Tumour classification: squamous cell carcinoma (25), adenocarcinoma (1), adenosquamous carcinoma (4). In addition, formalin-fixed, paraffin-embedded tissue blocks from patients with breast (n=5), stomach (n=5), colon (n=5), and prostate cancer (n=5) were used.

Cells, chemicals and antibodies

Human umbilical vein endothelial cells (HUVEC) were isolated[5]. Cells from passage 3 to 6 were grown in M199 medium (InVitrogen) containing 20% heat inactivated Fetal Calf Serum (Perbio), 0.05 mg/ml heparin, 2 mM Glutamine, 100 U/ml Penicillin, 100 µg/ml Streptomycin (InVitrogen) and 12.5 µg/ml Endothelial Cell Growth Supplement (Sigma-Aldrich) in fibronectin coated culture flasks (0.05 mg/ml). Human recombinant $VEGF_{165}$ was from Calbiochem. Antibodies: MMP-7 (clone 111433, IgG2B mouse monoclonal detecting pro and active MMP-7, R&D systems), CD34 (mouse monoclonal, Zymed), CD105 (mouse monoclonal, Dako).

Immunohistochemistry

Paraffin sections were stained as previously described[6]. Deparaffinized and rehydrated 4µm sections were quenched for endogenous peroxidase with 0.3% H_2O_2 in methanol before incubation with primary antibodies (o/n) diluted in phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA). Biotinylated rabbit anti-mouse immunoglobulins followed by horseradish peroxidase (HRP)-streptavidin complex (both Dako) were applied for 30 min each. Immune complexes were visualized using 0.05% diaminobenzidine (DAB, Sigma), containing 0.0038% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6) resulting in brown precipitate or alternatively Nova Red (Vector Laboratories, Burlingame, CA) resulting in red staining, and counterstained with Mayer's hematoxylin. Citrate antigen retrieval was used when indicated by the manufacturer. Appropriate tissue sections were included as positive controls and incubation with PBS-BSA without primary antibodies served as negative controls. MMP-7 staining in tumour and endothelial cells was independently scored by 2 individuals (C.S. and K.Z.), integrating the percentage of cells stained and the intensity of staining in a total score ranging from 0 to 8 [6]. A score from 0-4 was considered Low and 5-8 as High.

RNA isolation and real- time RT-PCR

Total RNA was isolated from HUVECs cultured on fibronectin, gelatin or collagen I coated 6 well plates with or without 100 ng VEGF per well, using the RNeasy Mini kit (Qiagen). Reverse transcriptase PCR was carried out in 1 µg RNA using random primers and a cDNA synthesis kit (Promega). MMP-7 expression was quantified using real-time quantitative PCR according to the TaqMan method (Applied Biosystems, Hs 00159163m1, Perkin-Elmer) with GAPDH as endogenous housekeeping gene[7]. Double-stranded MMP-7 cDNA was used as positive control.

Sprouting assay

Endothelial spheroids were prepared from HUVEC[8,9] in complete M199 medium containing 0.1% carboxymethylcellulose (Sigma-Aldrich) and 20% FCS, and seeded into non-adherent round bottom 96 well plates. After 24 h the spheroids were embedded in 1 mg/ml collagen matrix (Vitrogen 100, Nutacon) containing 20% FCS and treated with or without 100 ng/ml VEGF in complete M199 medium. Sprout-formation started after 3 hrs. Following overnight incubation, the spheroids containing collagen matrix was fixed in formalin and embedded in paraffin for immunohistochemical analysis.

MMP-7 activity assay

MMP-7 activity in cell culture media was determined using a recently developed immunocapture-based activity assay[10].

Immunoblot

Samples were analyzed on 15% SDS-PAGE under non-reducing conditions. Proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany). Blots were washed with PBS containing 0.05% Tween-20 (PBST, Merck) 3 times for 5 minutes. Non-specific binding was blocked with 0.2 % gelatin in PBST for 30 minutes at room temperature. MMP was detected by incubation with anti-MMP-7 antibody (IgG2B mouse monoclonal antibody, R&D), followed by biotinylated goat-anti-mouse antibodies and Streptavidin-HRP (both Dako). Coloration was performed using DAB.

Statistical analysis

Spearman correlations between parameters, survival curves, Log-Rank analyses and multivariate Cox analyses were performed using the SPSS 10.0 software package (SPSS Inc). *P* values ≤ 0.05 were considered significant.

Results

Representative stainings of MMP-7 in endothelial cells in respectively stomach, colon, breast, cervix, and prostate cancer tissue is shown in Figure 1A-E. The unexpected staining of MMP-7 in endothelial cells was confirmed using 3 other anti- MMP-7 antibodies with similar results (rabbit polyclonal RP1MMP-7 from Triple Point, rabbit polyclonal from Abgent and goat polyclonal from R&D, data not shown). The presence of MMP-7 expression in endothelial cells was associated with co-staining for CD34 and CD105 in sequential sections from the same tissue, suggesting that these cells were of neo-angiogenic origin (Figure 1). MMP-7 expression in endothelial cells was in general less intense than expression in epithelial cells. MMP-7 staining in endothelial cells did not correlate with the expression in epithelial cells in the same sections (R=0.069, P=0.716, n=50).

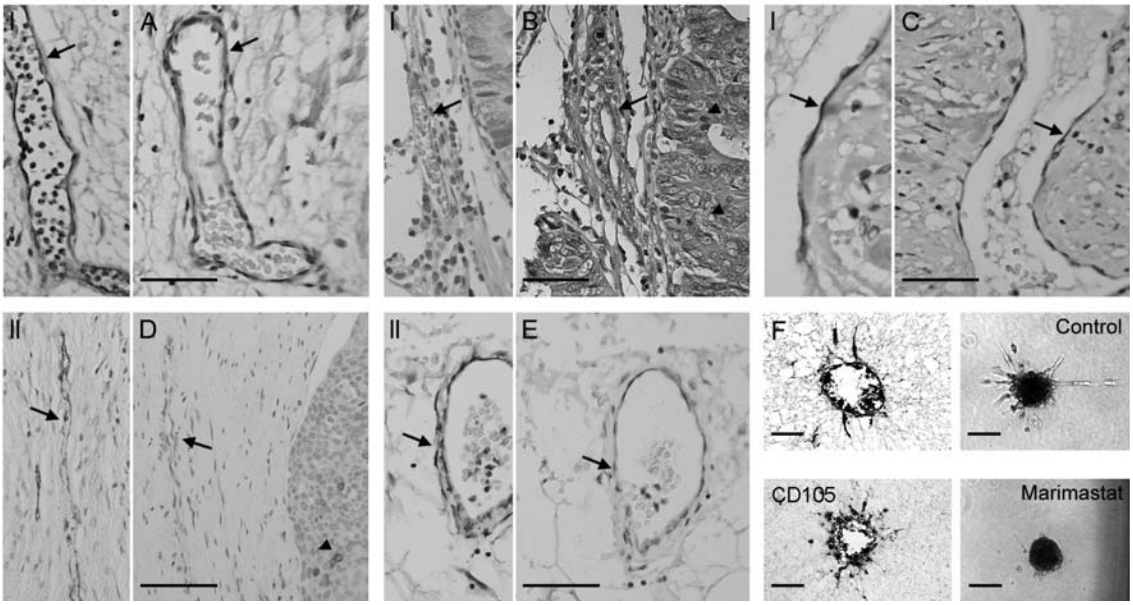

Figure 1. Immunohistochemical staining of endothelial cells for MMP-7, indicated by arrows in gastric cancer (A), colonic cancer (B), breast cancer (C), cervical cancer (D), prostate cancer (E), and in in vitro sprouting HUVEC cells (F). Inserts I and II indicate respectively CD34 and CD105 staining in sequential section from the same tissue. The inserts in (F) show VEGF-induced endothelial cell sprouting in control and marimastat-treated HUVEC spheroids. Arrowheads indicate epithelial cell staining. Bars correspond with 100 μm in (A-E) and with 300 μm in (F). Full-colour illustration at page 207.

MMP-7 expression is not expected to be present in endothelial cells. Therefore, we first demonstrated that endothelial cells are indeed able to express MMP-7 by evaluating MMP-7 mRNA levels in HUVEC. Unstimulated HUVEC, cultured on fibronectin, did not express detectable quantities of MMP-7 mRNA (> 40 cycles of RT-PCR). However, when grown on gelatin or collagen type I, HUVEC did express MMP-7 mRNA, and VEGF stimulation up-

regulated MMP-7 mRNA expression more than 3-fold after 16 hrs (comparative C_t method: dC_t 11.84 versus 10.43 respectively). Longer periods of VEGF stimulation resulted in decreasing levels of MMP-7 mRNA. Increased mRNA levels were reflected by the MMP-7 level in the conditioned medium. Serum-free medium from VEGF-stimulated HUVEC contained a similar level of MMP-7 as found in HT-29 colon cancer cell medium as quantified by a specific MMP-7 activity assay (24 versus 15 ng/ml respectively) and illustrated on the immunoblot in Figure 2 in medium and cell homogenates.

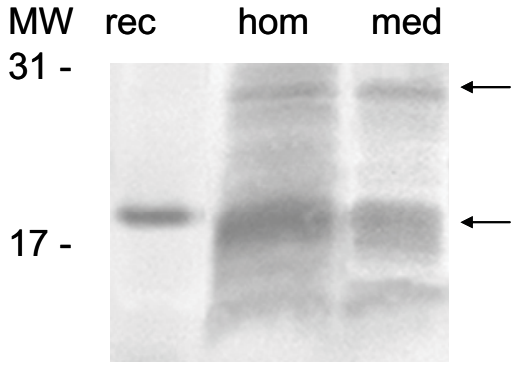

Figure 2. Immunoblot staining MMP-7 in endothelial cells and culture medium.
rec: recombinant active MMP-7; hom: homogenates of HUVEC grown on collagen type I and treated with 100 ng/ml VEGF; med: serum-free culture medium of HUVEC treated with VEGF. Arrows indicate presence of active (18 kDa) and pro-form (28 kDa) of MMP-7.

Subsequently, we used a 3-dimensional *in vitro* endothelial cell sprouting model to confirm our immunohistochemical and cell culture data. Figure 1F shows the presence of MMP-7 in VEGF-treated sprouting HUVEC. As expected, these cells stained also for CD105. Without VEGF stimulation HUVEC did not form sprouts and also stained less intense for MMP-7 and CD105 (data not shown). Addition of 1μg/ml Marimastat, a broad-range MMP inhibitor, prevented VEGF-mediated sprout formation completely (insert Figure 1F), suggesting the involvement of MMP activity in tumour angiogenesis.

We evaluated the relation of MMP-7 expression in endothelial cells as well as cancer cells from the 30 cervical cancer patients with the clinico-pathological parameters described in the *Patients, materials and methods* section. None of these parameters were significantly correlated with MMP-7, except for the expression in endothelial cells with survival (Low (score 0-4) versus High (score 5-8), Log Rank 5.12, P=0.02, Figure 3a).

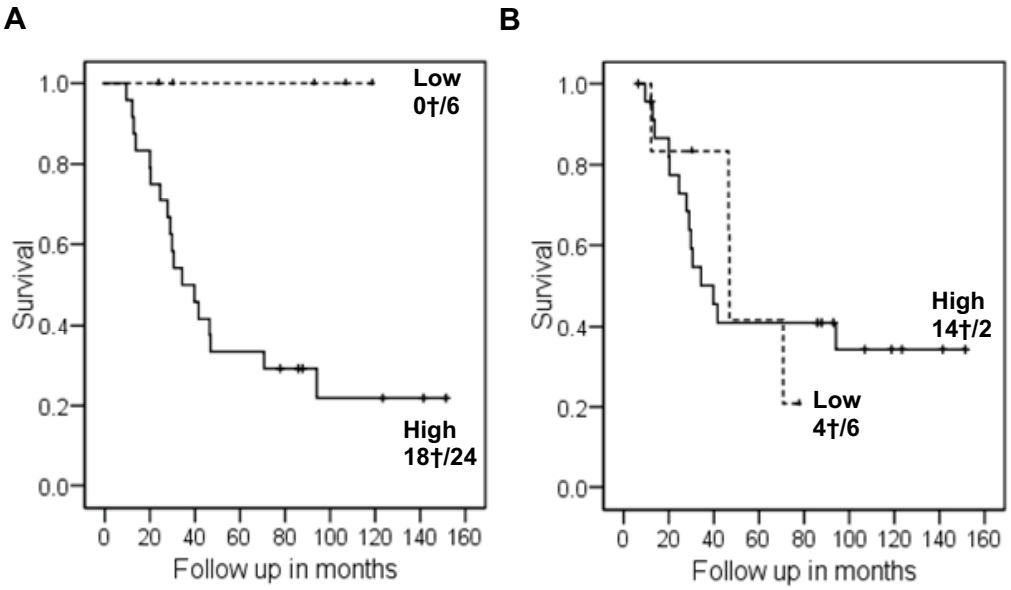

Figure 3. Kaplan-Meier survival curves for cervical cancer patients stratified for Low (score 0-4) and High (score 5-8) MMP-7 immunohistochemical staining. A) MMP-7 in endothelial cells (LR 5.12, P=0.02). B) MMP-7 in cancer cells (LR 0.00, P=0.99).

MMP-7 expression in tumour cells of these patients was not correlated with survival (Log Rank 1.91, P=0.17, Fig. 3b). In multivariate analysis against all the clinico-pathological parameters all the univariate significant parameters, i.e. endothelial MMP-7 staining, FIGO stage, infiltration depth, and parametrial invasion lost their statistical significance due to the clinico-pathological parameter distant metastasis (17 of 18 deceased patients had distant metastasis).

Discussion

Previously, MMP-7 has been found in early adenoma epithelial cells, in malignant cells at the invasive front, and in colon-derived liver metastases[11,12]. MMP-7 staining was also demonstrated in colonic endothelial cells adjacent to MMP-7 positive malignant epithelial cells, without detectable MMP-7 mRNA levels in endothelial cells[3]. Our results show the presence of MMP-7 in angiogenic endothelial cells in various cancer types. Endothelial MMP-7 expression was not dependent on the proximity of malignant MMP-7 expressing epithelial cells, suggesting an endogenous endothelial origin. RT-PCR data indicated that endothelial cells under angiogenic conditions are indeed able to express MMP-7. Moreover,

we have shown that under conditions closely resembling the *in vivo* neo-angiogenic process, MMP-7 up-regulation in endothelial cells coincides with sprout formation. Addition of Marimastat, a broad-range MMP inhibitor, prevented sprout formation completely without affecting cell viability. This suggests that endothelial cell-derived MMPs are involved in neo-angiogenesis. From all MMPs, especially MMP-7 could be of key importance in cancer, because it acts locally due to its cell membrane-adhering properties, secondly because it is able to activate other pro-MMPs like MMP-2, -8 and -9, and thirdly because of its capacity to release/cleave other important bioactive molecules[13]. The importance of MMP-7 in angiogenesis was underlined by the clinical data from our study, showing an association between endothelial MMP-7 expression and decreased survival in cervical cancer patients. Multivariate analysis did not reveal endothelial MMP-7 staining as a independent prognostic marker against clinico-pathological parameters, but this was probably due to the strength of one of these parameters in this small population: 17 of the 18 deceased patients had a distant metastasis. Support for endothelial MMP-7 as a prognostic indicator was recently provided by a study in 156 renal cell cancer patients[14].

In conclusion, MMP-7 expression was demonstrated in endothelial cells of various tumours and was associated with decreased survival in a cohort of cervical carcinoma patients. Considering the role of MMP-7 as local activator of proteinases and other biologically active proteins, specific inhibition of MMP-7 could contribute to inhibition of angiogenesis and anti-cancer therapy in general.

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