Cell-cell interactions in the gastrointestinal tumour-microenvironment

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Chapter 6

VEGF release by MMP-9 mediated heparan sulphate cleavage induces colorectal cancer angiogenesis

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Abstract

Angiogenesis is crucial for the progression of colorectal carcinomas in which bioavailability of Vascular Endothelial Growth Factor (VEGF) plays a major role. VEGF bioavailability is regulated by proteolytic release or cleavage. In colorectal cancer patients we observed a significant correlation between circulating VEGF and tumour tissue Matrix Metalloproteinase-9 (MMP-9) levels but not with MMP-2. Therefore, we evaluated the role of MMP-9 in regulating VEGF bioavailability and subsequent angiogenesis in 3-dimensional human cell culture models. MMP-9 treatment released VEGF dose-dependently from HT29 colon carcinoma spheroids, comparable to heparitinase, a known mediator of VEGF release. Conditioned medium from human neutrophils, containing high amounts of active MMP-9, released VEGF comparable to recombinant MMP-9, in contrast to myofibroblast medium**.** MMP-9 treated spheroids showed decreased extracellular levels of heparan sulphates, required for VEGF binding to the matrix, whereas the levels in the medium were increased. Western blot analysis revealed that $VEGF_{165}$ is the major isoform released by MMP-9 treatment. *In vitro* experiments indicated that MMP-9 is not capable to cleave $VEGF_{165}$ into smaller isoforms, like plasmin does. These data suggested that MMP-9 mediates release rather than cleavage of larger VEGF isoforms. Medium from MMP-9 treated HT29 spheroids induced endothelial cell sprouting in an angiogenesis assay, comparable to the effect of recombinant $VEGF_{165}$. Anti-VEGF antibody treatment resulted in a strongly reduced number of sprouts. In conclusion, we have shown that neutrophil derived MMP-9 is able to release biologically active $VEGF_{165}$ from the ECM of colon cancer cells by cleavage of heparan sulphates.

Introduction

Colorectal carcinomas (CRC) are characterized by enhanced VEGF expression and corresponding high microvascular densities, indicating increased angiogenic activity and leading to worse patient survival[1,2]. Therapy using anti-VEGF antibodies improves CRC patient survival, emphasizing VEGF as a major angiogenic factor[3-6]. VEGF expression is up-regulated by hypoxia and various tumour-related cytokines including Transforming Growth Factor-β, Interleukin-1β, Platelet Derived Growth Factor and Epidermal Growth Factor[7,8]. At least six human VEGF isoforms are known, ranging in length from 121 to 206 amino acids, from which $VEGF_{165}$ is the most predominant. Except for $VEGF_{121}$ all isoforms contain a heparin-binding domain mediating adhesion to the extracellular matrix (ECM) by interaction with heparan sulphate proteoglycans (HSPGs)[9]. Binding of larger isoforms to the ECM provides a reservoir of biologically active VEGF. Consequently, the local release of soluble VEGF is a key factor in angiogenesis. Various proteinases have been studied as mediators of VEGF cleavage and/or release, including members of the matrix metalloproteinase (MMP) family like MMP-2[10,11], MMP-7[12], MMP-9[13] and MMP-14[14,15]. Most of the experiments showing the involvement of proteinases in VEGF-release were done using animal models and simple *in vitro* systems. Because interspecies differences have been described for several substrate/MMP combinations[16], we investigated the role of the gelatinases MMP-2 and MMP-9 in VEGF-bioavailability in human CRC. First, the concentration and localization of VEGF, MMP-2 and MMP-9 were determined in tissues and plasma of CRC patients. Next, we used a 3-dimensional ECM-producing human colon cancer model system to evaluate the role of several MMPs in VEGF release. Recombinant MMPs and conditioned media from neutrophils and myofibroblasts, cell-types associated with the angiogenic switch, were evaluated for their MMP content and VEGF-releasing capacity. VEGF functionality was determined in a 3-dimensional human endothelial cell-sprouting assay, resembling *in vivo* angiogenesis. This study shows the importance of especially MMP-9 in the release of biologically active $VEGF_{165}$ from the ECM, mainly by cleavage of HSPGs, leading to the angiogenic switch in CRC.

Materials and methods

Patient material

Pre-operative plasma and tissue specimens from 46 patients (29 ♂, 17 ♀) undergoing resection for primary CRC at the department of Oncologic Surgery, Leiden University Medical Centre, were collected as described before[17]. Tissues were homogenized in Tris/Tween-80 and the protein concentrations were determined as described previously[18,19]. For immunohistochemistry, tissue specimens were fixed in 4% paraformaldehyde, dehydrated in graded alcohol and xylene and embedded in paraffin. All human samples were used according to the guidelines of the Medical Ethics Committee of the Leiden University Medical Centre.

VEGF ELISA

VEGF levels in tissue, plasma and cell culture supernatants were determined using an ELISA (DY293b, R&D Systems Europe, Abingdon, UK) in combination with a substrate reagent pack (DY999) according to the manufacturers' instructions, detecting specifically human $VEGF_{121}$ and $VEGF_{165}$.

MMP -2 and -9 ELISAs

MMP-2 and MMP-9 levels were determined by ELISAs as described before[20]. In short, plates were coated with anti-MMP-2 or anti-MMP-9 as capture antibodies and incubated with appropriately diluted samples (o/n, 4°C). Polyclonal rabbit anti-MMP-2/biotin-labelled goat anti-rabbit-IgG and biotin-labelled polyclonal anti-MMP-9 antibodies were used for immune-detection. Colour development was performed with streptavidin-peroxidase/tetramethyl benzidine/H_20_2. The reaction was stopped with 1M H_2SO_4 and the absorption was measured at 450 nm. Sample concentrations were calculated in ng/mg protein. ρ-Aminophenyl-mercuric acetate (APMA) was used to activate pro-MMP-9 (in 0.25 mM in 50 mM Tris-HCl, pH7.6, 1.5 mM NaCl, 0.5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.01% Brij35, 1.5 hours at 37º). MMP-9 activation was monitored by zymography as before[18].

Immunohistochemistry

Immunohistochemistry on 5 μm paraffin sections was performed as described before[21]. Sections were incubated overnight at room temperature with primary unlabelled antibodies (Table 1). Frozen 4 μm sections for HSPG staining were fixed in ice-cold acetone for 10

minutes and incubated with monoclonal mouse anti-HSPG antibodies (10E4 kindly provided by Prof G. David, Leuven[22]) overnight at 4ºC. Detection of primary antibodies was performed as before[21]. Human placenta was used as a positive control for all antibodies. Negative controls were included by omitting the primary antibodies. Representative photomicrographs were taken with a Leica DMLB microscope and DC500 camera.

Table 1. Antibodies used for immunohistochemistry

Antibody	**Clone**	**Dilution**	**Supplier**	***Antigen retrieval***
CD31	JC70A	1:400	Dako, Glostrup, Denmark	Citrate[1]
CD34	B1-C35	1:1600	Zymed, San fransisco, USA	Citrate
Vimentin	V-9	1:1000	Santa Cruz, Biotechnologies, Santa Cruz, USA	None, cytospins
SMA	ASM-1	1:1500	Progen, Heidelberg, Germany	None, cytospins
		1:1000		Citrate
VEGF	rabbit polyclonal	1:400	Santa Cruz, Biotechnologies, Santa Cruz, USA	Citrate
MMP-2	rabbit polyclonal	1:250	TNO, Leiden, the Netherlands	Citrate
MMP-9	rabbit polyclonal	1:400	TNO, Leiden, the Netherlands	Citrate
HSPGs	10E4	1:800	Prof. G. David, Leuven	Frozen sections
Laminin	rabbit polyclonal	1:800	Abcam, Cambridge, UK	Frozen sections
CD105	SN6h	1:1000	Dako, Glostrup, Denmark	None[2]

[1] *10 minutes boiling in 0.01 M citrate buffer, pH 6.0*

[2] *Catalysed signal amplification system used according to manufacturers' protocol (Dako)*

Cell culture and preparation of spheroids

HT29 colon carcinoma and fibroblast spheroids were prepared as described before (2,500 cells/well)[23] and collected after 48 hour of incubation. For immunohistochemistry the spheroids were fixed in 4% formaldehyde overnight at 4°C, dehydrated and embedded in paraffin. Human umbilical vein endothelial cells (HUVECs) were isolated according to Jaffe

and colleagues[24] Cell culture and spheroid generation were performed as described before[25]. After 24 hours the spheroids were collected for the collagen sprouting assay.

Treatment of HT29 spheroids with MMPs and heparitinase

HT29 spheroids were incubated in 48 well plates (8-10 per well), coated with 0.6% agarose, in 100 µl serum free (SF)-DMEM/F12 medium containing 0-128 ng/ml activated recombinant human (rh) MMP-2 (kindly provided by TNO, Quality of life BioSciences), rh-MMP-7 (R&D-systems), rh-MMP-8 (Chemicon Europe, Chandlers Ford, UK) or rh-MMP-9 (Invitek, Berlin, Germany), 128 ng/ml MMP-9 with 1 µg/ml Marimastat, a broad range MMP-inhibitor, kindly supplied by British Biotech Pharmaceuticals, or 5 mU heparitinase (Seikagaku Corporation, Tokyo, Japan). Pro-MMPs were activated as described above. After 24 hours incubation at 37°C, conditioned media (CM) were collected and applied to the collagen sprouting assay. The VEGF levels were determined by ELISA. VEGF isoform determination was performed by incubating 200 HT29 spheroids in 100 µl MMP-9 containing SF-DMEM/F12 to obtain VEGF levels above western blot detection limit. HT29 spheroids were embedded in OCT and frozen in liquid nitrogen for HSPG staining.

Western blots for VEGF and HSPGs

Samples were analysed on 10-15% SDS-PAGE under reducing and non-reducing conditions. Proteins were transferred to a nitrocellulose membrane (Whatman, Dassel, Germany) overnight. After each step blots were washed 3 times with PBS containing 0.05% Tween-20 (PBST, Merck). Non-specific binding was blocked with 0.2 % gelatin in PBST for 30 minutes. VEGF was detected by incubation with polyclonal rabbit-anti-human VEGF antibody (Santa Cruz) recognizing VEGF isoforms 121, 165 and 189, followed by biotinylated goat-anti-rabbit antibodies and streptavidin-HRP (both Dako). HSPGs was detected with mouse monoclonal antibody 3G10 recognizing HSPG stubs (cleaved heparan sulphates) followed by goat anti-mouse HRP[22]. Detection was performed using Super Signal West according to the manufacturers' protocol (Pico Chemoluminescent substrate, Pierce, Rockford, IL, USA).

Collagen sprouting assay

HUVEC spheroids were embedded in 96 well plates in type I collagen matrix consisting of basic M199 medium supplemented with 20% FCS and 1 mg/ml collagen (Type I, calf skin, Vitrogen, Palo Alto, USA). After 1 hour of polymerisation, 75 µl of the medium from the

treated HT29 spheroids was applied. After overnight incubation sprout-formation was determined by counting the number and average length per spheroid (double blind by 2 individuals). Complete M199 medium with or without 100 ng/ml recombinant human (rh)-$VEGF_{165}$, (Calbiochem, La Jolla, CA, USA), and active MMP-9 which was not incubated with HT29 spheroids were included as controls. To confirm the role of VEGF, MMP-9 treated HT29 spheroid CM was supplemented with anti-VEGF antibody (Bevacizumab/Avastin), kindly provided by Dr. I.M. Teepe-Twiss).

Preparation of neutrophil and myofibroblast CM

Neutrophils were isolated from 80 ml whole human blood using a Ficoll gradient. Remaining erythrocytes were lysed with 160 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.4. Neutrophils ($6x10^6$/ml) were incubated in SF-RPMI 1640 medium (Invitrogen) for 24 hours under argon to induce hypoxia, resulting in the release of MMP-9.

Human CRC fibroblasts were isolated by outgrowth of the cells from resection specimens. Fibroblast origin was confirmed by morphology, vimentin staining (>95% positive) and ~50% α-smooth muscle actin (SMA) staining. Fibroblasts were maintained in 10% DMEM/F12 and used at passage 5-11. Myofibroblasts were generated by stimulation with 5 ng/ml TGF-β1 (Peprotech, United Kingdom) overnight. Differentiation into myofibroblasts was confirmed by SMA-staining. Medium was changed to SF-DMEM/F12 and collected after 24 hour incubation. All CM were analyzed for MMP-2 and -9 content and activity by zymography.

Proteolytic cleavage $VEGF_{165}$

To analyze the capacity of MMP-9 to cleave $VEGF_{165}$ *in vitro* 1 µg rh-$VEGF_{165}$ (Calbiochem) was incubated in MMP-9 activation buffer with/without 1280 ng/ml active MMP-9 or with 0.1 U/ml human plasmin (Sigma-Aldrich) in PBS. After incubations of 30 minutes, 4h and overnight samples were analysed by western blot.

Statistical analysis

Statistical analyses were performed using SPSS Statistical Package 11.0 (SPSS Inc., Chicago, USA). Differences between normal and tumour levels were calculated using the Wilcoxon signed rank test. Sprout formation data are presented as mean ± standard deviation and extent of endothelial cell sprout formation was analysed using the Student's *t*-test. For the correlation between tissue and serum levels Pearson's correlation analysis was used. $P \leq 0.05$ was considered significant.

Results

VEGF and MMP-2 and -9 levels in CRC and in the circulation

Figure 1 shows the concentrations of VEGF and MMP-9 in CRC and corresponding mucosa. VEGF and MMP-9 levels were significantly enhanced in tumours (both $P<0.0001$, n=46 pairs) and were mutually correlated (R=0.405, $P<0.0005$, n=92). Tissue MMP-2 levels were not enhanced in CRC compared to normal mucosa (18.4 versus 18.3 ng/mg protein, n=46 pairs) and did not correlate with the tumour VEGF concentration. Tumour levels of MMP-2 and MMP-9 correlated weakly with each other (R=0.250, $P=0.019$), but only MMP-9 level correlated significantly with the circulating VEGF concentration (MMP-9: R=0.379, $P=0.009$; MMP-2: R=-0.052, $P=0.729$, n=46). Circulating VEGF was not correlated with tumour VEGF levels (R=-0.048, $P=0.754$).

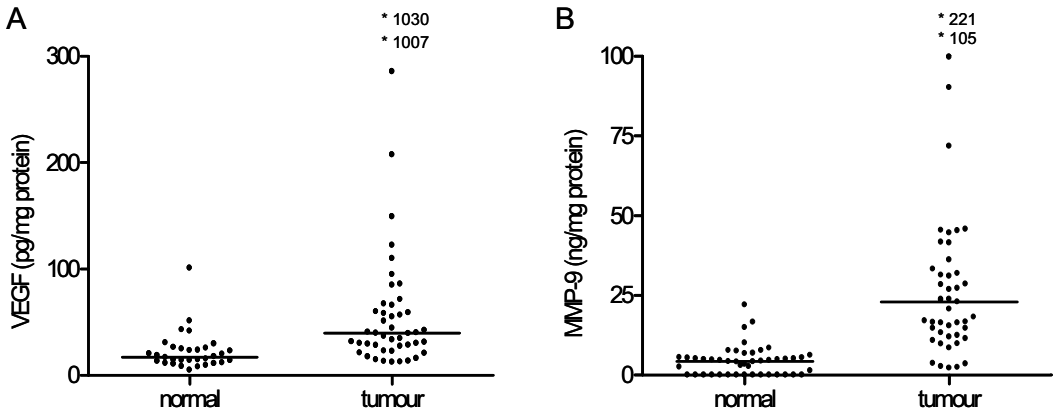

Figure 1. Protein levels of VEGF and MMP-9 in tissues from 46 colorectal cancer patients. In cancer samples VEGF (A) and MMP-9 (B) levels are significantly (P<0.0001) increased compared to corresponding normal tissue. Horizontal lines indicate median values.

To examine the association of tumour MMP-9 and VEGF release, we performed immunostaining in consecutive sections. In normal colon tissue, weak VEGF staining was detected in epithelial cells (Figure 2A). MMP-9 was detected in some endothelial cells of small blood vessels and in infiltrating leukocytes, although the frequency of these cells was low (Figure 2D). In CRC, high VEGF expression was detected exclusively in epithelial cells and endothelial cells (Figure 2B). MMP-9 was observed in SMA-positive myofibroblasts (Figure 2E), infiltrating leukocytes, especially neutrophils (insert Figure 2E), and weakly in some tumour cells. All endothelial cells stained for CD31, whereas markers for newly formed blood vessels (CD34 and CD105) were restricted to small blood vessels in the submucosal

area in normal mucosa, but were rarely observed in the proximity of epithelial cells (Figure 2I). Strongly increased numbers of CD34 and CD105 positive vessels were present in CRC, mainly localized in between tumour cells (Figure 2J).

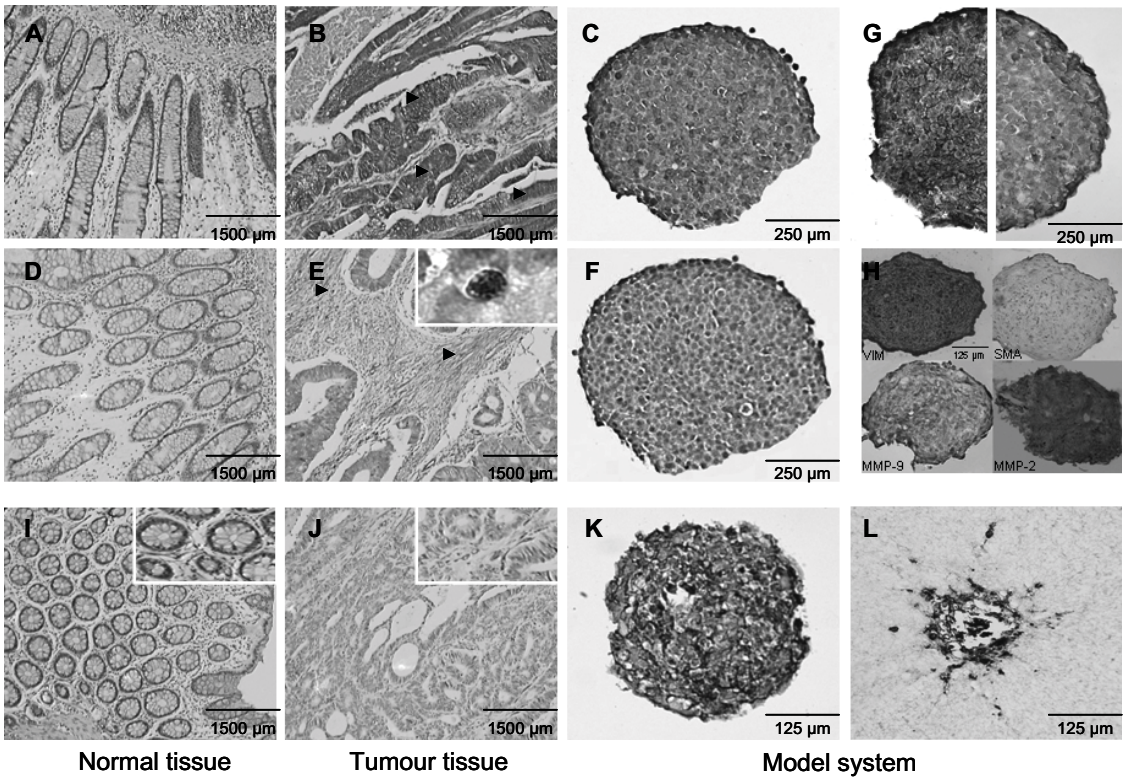

Figure 2. Immunohistochemical staining of normal and tumour colon tissues and 3-dimensional cell culture models showing the resemblance between patients derived tissue and in vitro cell culture models. VEGF expression was very low in normal colon tissue (A) and strongly increased in colon tumour cells (B, arrowheads). HT29 colon cancer spheroids strongly express VEGF (C). MMP-9 was hardly detectable in normal colon tissue (D) and strongly expressed in stromal cells in colorectal cancer (E), especially in neutrophils (enlarged insert, 630x). HT29 spheroids were negative for MMP-9 (F), and produced VEGF binding HSPGs (G, left) and the ECM molecule laminin (G, right). Fibroblast spheroids (H) were positive for vimentin (VIM) and for SMA. MMP-9 was expressed, but MMP-2 was more abundant. CD34 showed minor staining in normal colon except for the submucosal area (I, insert). In tumours many neo-angiogenic CD34 positive vessels are present in the proximity of tumour cells (J, insert, 630x). HUVEC spheroids were positive for CD31 (K) while sprouting endothelial cells showed additional positivity for the angiogenesis marker CD105 (L). Full-colour illustration at page 206.

Characterization of HT29, fibroblasts and HUVEC spheroids

To validate the resemblance of the cell culture models with human CRC tissue, HT29, fibroblasts and HUVEC spheroids were stained for VEGF, MMPs, cell-markers and ECM molecules. Strong expression of VEGF in HT29 spheroids was observed (Figure 2C), whereas

virtually no MMP-9 (Figure 2F) or MMP-2 (not shown) expression was present, consistent with the findings *in vivo*. HT29 spheroids contained functional ECM components, including HSPGs (Figure 2G, left) and laminin (right). Fibroblast spheroids showed strong vimentin expression, and were positive for SMA. MMP-9 was expressed, but MMP-2 expression was more abundant. (Figure 2H) HUVEC spheroids stained for CD31 (Figure 2K) and sprouting HUVECs additionally showed positive staining for CD105 (Figure 2L).

MMP mediated VEGF release from HT29 spheroids

To assess whether MMPs were capable of releasing VEGF from colorectal cancer ECM, HT29 spheroids were treated 24 hours with active rh-MMP-2, -7, -8, or -9. Medium from untreated HT29 spheroids contained low levels of VEGF, which was dose dependently 3-fold increased by treatment with 32-128 ng/ml MMP-9 (Figure 3A).

Treatment with MMP-7 or low concentrations of MMP-2 or MMP-8 did not influence VEGF release. Treatment with high concentrations of MMP-2 or MMP-8 led to maximal 2-fold VEGF increase. Addition of MMP inhibitor Marimastat reduced MMP-9-mediated VEGF release to basal levels (Figure 3A). Control media containing APMA did not affect VEGF release (not shown). Treatment of HT29 spheroids with neutrophil-derived CM, containing high amounts of active MMP-9 (zymogram Figure 3B), resulted in release of VEGF levels comparable to treatment with 128 ng/ml rh-MMP-9 (Figure 3C). Marimastat inhibited the release significantly.

Treatment of the spheroids with myofibroblast CM, containing high amounts of MMP-2 and minor amounts of MMP-9 (zymogram Figure 3B), resulted only in a mild increase in VEGF, comparable to 128 ng/ml MMP-2 treatment, which was completely inhibited by Marimastat. The MMP-9-mediated VEGF release was comparable to the effect of heparitinase (Figure 3A), a HSPG digesting enzyme known to release VEGF[7].

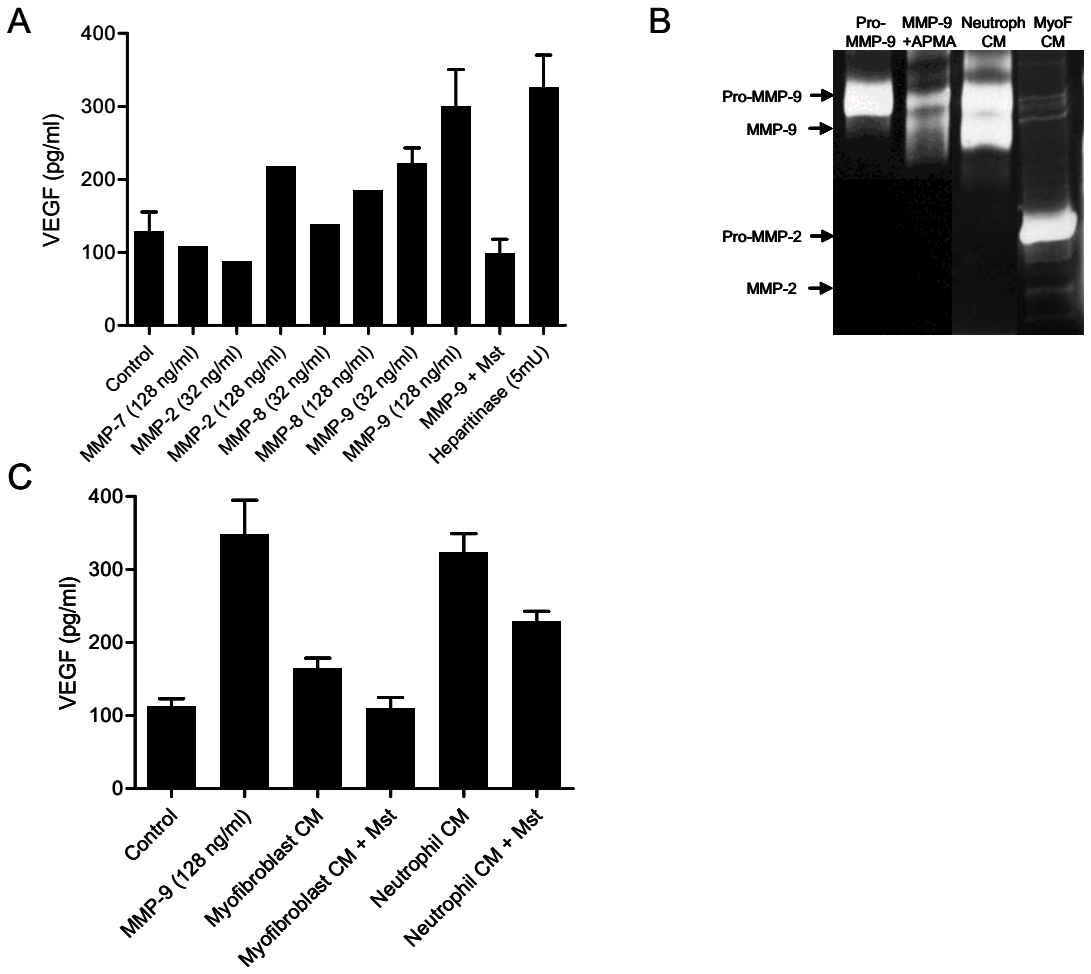

Figure 3. A) VEGF release (mean±SEM) from colon carcinoma spheroids after treatment with various MMPs and heparitinase as control. HT29 colon carcinoma spheroids were treated for 24 hours with 0, 32 or 128 ng/ml active MMP-2, -7, -8 or -9. Treatment with MMP-9 resulted in the highest dose-dependent release of VEGF, completely inhibitable by addition of MMP inhibitor Marimastat (Mst, 1 µg/ml). The experiments were performed 3-6 times, except where no error bars are indicated (result of 3 pooled experiments). B) Gelatin zymogram showing respectively pro-MMP-9, APMA activated MMP-9 and conditioned medium (CM) from neutrophils and myofibroblasts. Neutrophil CM contained high levels of MMP-9, whereas myofibroblast released high levels of MMP-2 and low but detectable levels of MMP-9. C) VEGF release from HT29 spheroids treated for 24 hours with neutrophil and myofibroblast conditioned medium. The experiments were performed 3 times. MST: addition of 1 µg/ml Marimastat; CM: conditioned medium.

HSPG immunostaining on spheroids treated with MMP-9 or heparitinase showed a similar decrease in the extracellular localization of HSPG (Figure 4A), suggesting cleavage of HSPGs by MMP-9. To evaluate the effect of MMP-9 on HSPGs, 200 HT29 spheroids were treated with/without 128 ng/ml MMP-9 and the medium was subsequently immunoblotted with 3G10, a monoclonal antibody specifically recognizing heparitinase-treated HSPG

(Figure 4B). MMP-9 treatment resulted in stronger and additional bands compared with the controls, indicating release/cleavage of various types of HSPGs.

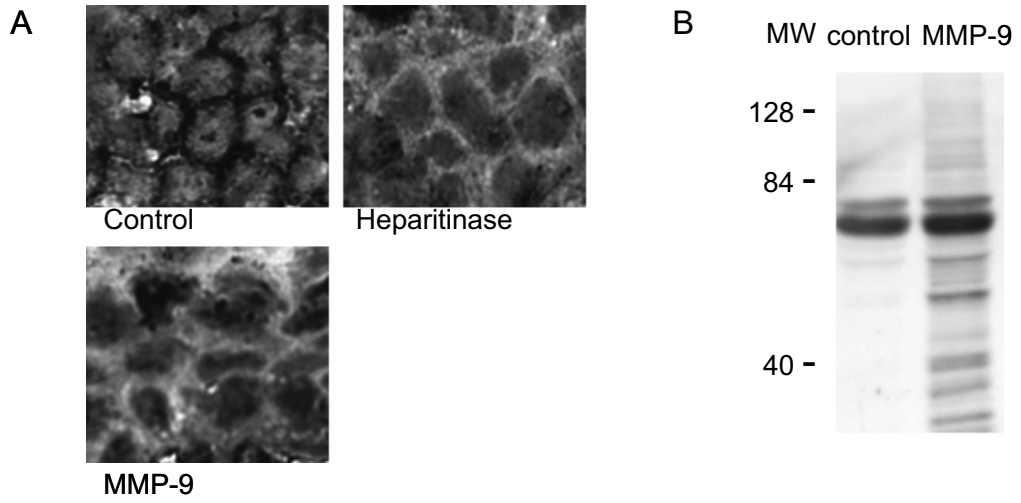

Figure 4. A) Immunohistochemical staining for HSPGs with monoclonal antibody 10E4 of HT29 spheroids after treatment with MMP-9 or heparitinase resulting in decreased extracellular localization of HSPGs (magnification 630x). B) Western blot of medium from HT29 spheroids treated with MMP-9 and subsequently stained with monoclonal antibody 3G10, specifically recognizing epitopes on HSPGs after cleavage. Full-colour illustration at page 207.

To examine whether MMP-9 cleaves $VEGF_{165}$ to smaller isoforms or that alternatively intact $VEGF_{165}$ is released, we incubated rh-$VEGF_{165}$ with 1280 ng/ml MMP-9 or plasmin, known to cleave $VEGF_{165}$ [9]. As shown in Figure 5A plasmin cleaves $VEGF_{165}$ resulting in a 17 kDa fragment after 4h. In contrast MMP-9 is not capable of cleaving $VEGF_{165}$ after 4h or even overnight incubation, indicating that MMP-9 mediates VEGF release rather than inducing cleavage. Western blot analysis of HT29 spheroid CM showed that $VEGF_{165}$ is the major isoform in the medium after MMP-9 treatment (Figure 5B).

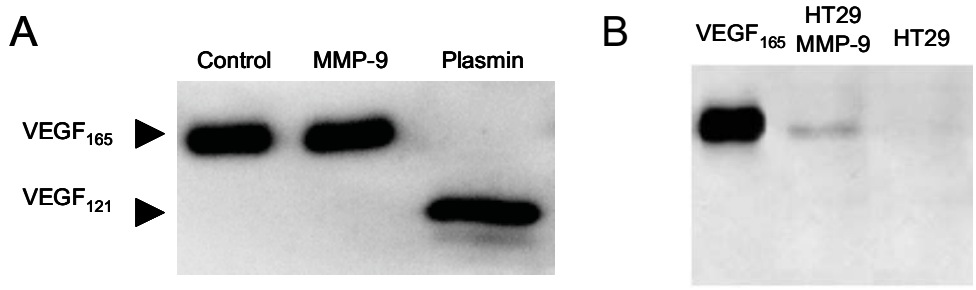

Figure 5. A) Western blot analysis (reduced gel) showing treatment of human recombinant $VEGF_{165}$ by MMP-9 and plasmin. Plasmin cleaves $VEGF_{165}$ to $VEGF_{121}$ whereas MMP-9 has no effect. Control: rh-$VEGF_{165}$; MMP-9: addition of 1280 ng/ml rh-MMP-9, 4h; Plasmin: addition of 0.1 mU human plasmin, 30 min).
B) Western blot analysis (unreduced gel) showing that after MMP-9 treatment of HT-29 spheroids $VEGF_{165}$ is the major isoform observed in the medium.
VEGF: 20 ng rh-$VEGF_{165}$; HT29/MMP-9: medium from HT29 spheroids treated with 128 ng/ml MMP-9; HT29: spheroid medium without treatment

HT29 spheroid-derived VEGF mediates endothelial cell sprouting

To determine if the released VEGF was capable of inducing endothelial sprouting we used a 3-dimensional angiogenesis model. Stimulation of HUVEC spheroids with untreated HT29 spheroid CM resulted in minimal endothelial sprout-formation, reflecting the low VEGF levels as determined by ELISA (Figure 6A) and comparable to the medium control (Figure 6B). In contrast, when HUVEC spheroids were incubated with medium from HT29 spheroids treated with 128 ng/ml MMP-9 extensive sprout formation was observed (Figure 6C), comparable to stimulation with 100 ng/ml rh-VEGF (Figure 6D). Analysis of the sprouts showed a significant increase in both length and number after stimulation with MMP-9-treated HT29 spheroid CM (Figure 7A/B). Medium from HT29 spheroids treated with MMP-9 in the presence of Marimastat did not result in endothelial cell sprouting (Figure 6E). Active MMP-9 itself was not capable of inducing sprout formation (Figure 6F).

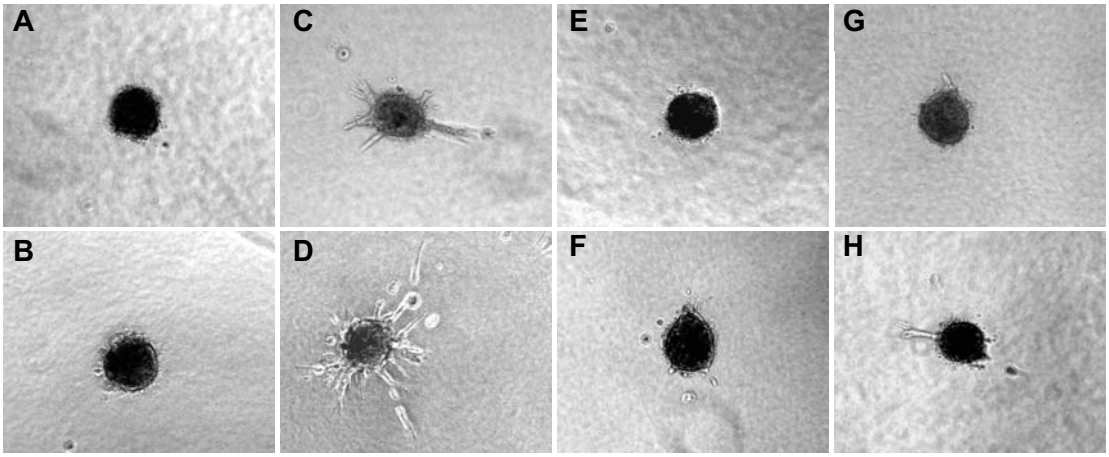

Figure 6. Sprout formation by MMP-9-released VEGF in a 3-dimensional angiogenesis model. Medium from untreated HT29 spheroids did not induce sprout formation in HUVEC cells (A), compareble to the medium control (B). The medium from MMP-9 treated HT29 spheroids strongly induced HUVEC sprout formation (C) comparable to rh-VEGF$_{165}$ (D). Treatment of HT29 spheroids with MMP-9 in the presence of Marimastat (Mst) resulted in strongly reduced sprout formation in HUVEC cells (E). Active MMP-9 itself was unable to induce HUVEC sprout formation (F). Sprout formation induced by VEGF$_{165}$ was strongly reduced by addition of Bevacizumab (G). Bevacizumab reduced the number of sprouts induced by MMP-9 treated HT29 spheroid medium, whereas sprout length was reduced to a lesser extent (H).

The contribution of VEGF in the angiogenesis assay was determined by pre-incubation with the neutralizing VEGF antibody Bevacizumab. Bevacizumab efficiently inhibited endothelial cell sprouting by rh-VEGF (Figures 6G and 7A/B). When incubated with MMP-9-treated HT29 spheroid CM, Bevacizumab inhibited the number of endothelial sprouts formed significantly (Figures 6H and 7A), whereas the reduction of the length of the residual sprouts was less pronounced (Figure 7B).

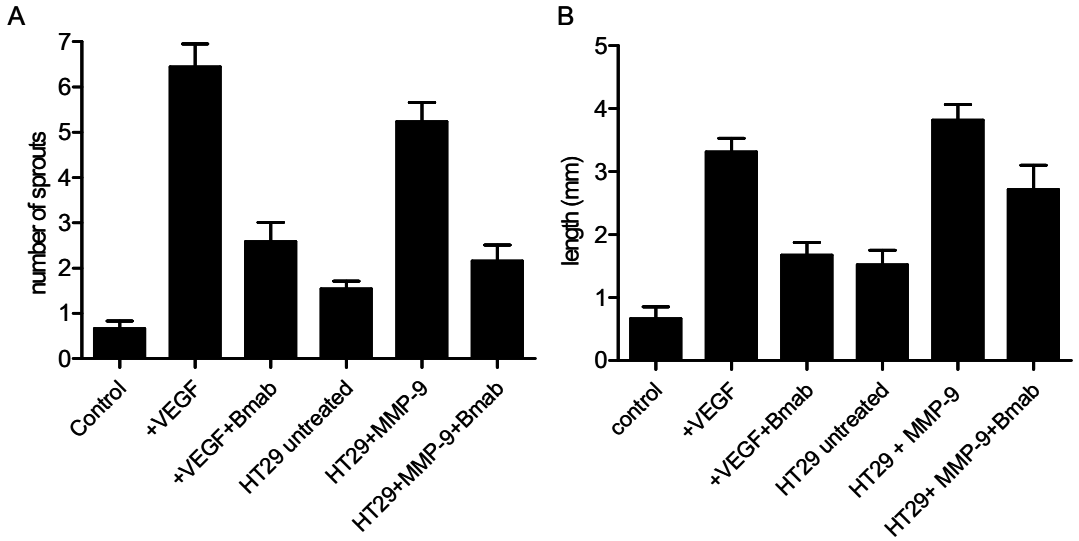

Figure 7. Quantification of number and length of sprouts induced by VEGF. The number of sprouts is significantly induced by VEGF, and is inhibited by Bevacizumab (Bmab). MMP-9 treated HT29 spheroid medium showed, like VEGF, significant induction of sprout formation, which is reduced to control level by addition of Bmab. The average sprout length was increased by VEGF which was found to be inhibited by Bmab. A comparable induction was observed by MMP-9 treated HT29 spheroids medium, but surprisingly the length of the sprouts could not completely be inhibited by the addition of Bmab.

Discussion

The ability of VEGF to induce angiogenesis depends on the presence of active, mobile isoforms within the microenvironment. After production and release from the cells, VEGF bioavailability is regulated via cleavage and/or release by proteolytic activity in combination with the acidic pH present in the tumour-microenvironment[26]. Heparanases[27,28], plasmin[9,29], urokinase[30,31], phoshatidyl-inositol phosholipase C[9], and MMPs[11-15] have been shown to cleave larger VEGF isoforms into smaller fragments or, alternatively, mediate release of VEGF by remodelling of the ECM. In our study, significantly increased and correlating VEGF and MMP-9 tumour levels were accompanied by a significant correlation of tumour MMP-9 and pre-operative circulating levels of VEGF, a factor associated with poor outcome in CRC-patients[32]. Tumour tissue MMP-2 did not correlate with circulating VEGF. These data suggested a prominent role for MMP-9 in the release and bioavailability of VEGF in CRC *in vivo*. MMP-9 has been shown to mediate VEGF release in mouse pancreatic cancer, whereas MMP-2 and urokinase had no effect[13]. Studies with human ovarian tumours xenografted in nude mice showed that MMP-2 and MMP-9 induced the release of VEGF in

ascites[11,33]. Those studies however did not distinguish whether VEGF was released via cleavage of larger VEGF isoforms or by ECM remodelling, leaving VEGF intact.

To elucidate the role of MMP-9 on VEGF release and angiogenesis in human CRC, we used 3-dimensional human cell-models, comparable to human tumour xenografts in mice[34], but avoiding inter-species complications. Our data show that MMP-9 induced VEGF release was comparable to the effect of the HSPG degrading enzyme heparitinase. In comparison, MMP-2 and MMP-8 were also capable of VEGF release, albeit less potent. Staining of MMP-9-treated spheroids showed a decrease in the extracellular localization of HSPGs, suggesting that MMP-9 cleaved HSPGs rather than VEGF itself, similar to what was found previously for VEGF bound to connective tissue growth factor[35]. Western blotting of medium from HT29 spheroids with monoclonal antibody 3G10 confirmed MMP-9 mediated cleavage of HSPGs.

Our *in vitro* data showed that in contrast to plasmin, human MMP-9, even after overnight incubation, did not cleave human $VEGF_{165}$ into a 17 kDa $VEGF_{121}$ fragment, as previously described for mouse VEGF[36], suggesting that in an) entirely human setting intact $VEGF_{165}$ release by MMP-9 is more probable than cleavage. Western blot analysis confirmed that the major isoform released from the HT29 spheroids is $VEGF_{165}$ rather than $VEGF_{121}$. Additionally, sequence alignment of mouse versus human $VEGF_{165}$ revealed a low homology in the expected cleavage sequence, presumably resulting in a different proteolytic sensitivity[37].

Immunohistochemistry on human CRC showed expression of VEGF primarily in tumour cells, whereas MMP-9 expression was mainly detected in stromal cells including neutrophils, endothelial cells, myofibroblasts, and incidental macrophages. *In vitro* myofibroblasts secreted mainly MMP-2 and low levels of MMP-9. Treatment with myofibroblast CM lead to increased VEGF release, probably induced by a combination of MMP-9 and MMP-2, but never reached the level of neutrophil CM. Tumour-infiltrating neutrophils have previously been shown to mediate the initial angiogenic switch in a model of multistage carcinogenesis[38]. In neutrophils, MMP-9 is located in granules and is not used for ECM-degradation during migration to the tumour, but is released upon signals from the tumour into the microenvironment without accompanying TIMP, resulting in high catalytic MMP-9 activity[39,40]. Therefore, extravasated neutrophils are good candidates to initiate rapid changes in the hypoxic microenvironment compared to other MMP-9 positive stromal cells. Besides MMP-9 neutrophils contain a range of other proteinases, which might contribute to VEGF-release or degradation. Previously we showed in gastric carcinoma homogenates that the level

of MMP-9 correlated significantly with myeloperoxidase, a specific neutrophil marker[41], suggesting that neutrophils are a major source of MMP-9. MMP-9 levels were also highly correlated with MMP-8, also called neutrophil collagenase. Although MMP-8 was less efficient than MMP-9 in this study, it could still be involved in VEGF release. Because MMP-8 resides in other granula in neutrophils than MMP-9 the time and/or the location of release of these proteinases presumably differs. Due to these variations in localization and activity levels within the tumours, even proteinases with lower efficiencies than MMP-9, including other MMPs could be involved in VEGF release in situ[10-12,14,15]. Our data using broad range MMP inhibitor Marimastat support this notion.

The involvement of MMP-9 in VEGF-mediated endothelial cell sprouting was confirmed by the use of the VEGF inhibitor Bevacizumab, resulting in inhibition of the number and to a less extend the length of endothelial sprouts. Variation in sprouting phenotype was previously associated to different $VEGF_{165}$ concentrations[42]. In our model it is more likely caused by other angiogenic factors present in the MMP-9-treated spheroid CM. These factors, like bFGF or TGF-β, are capable of stimulating endothelial sprout-outgrowth after sprouting has been initiated. Our data support the involvement of VEGF particularly in the onset of the angiogenic switch. Summarized in figure 8, we propose that in small, not yet vascularised tumours hypoxia leads to increased epithelial cell VEGF production, which stays bound to the ECM. MMP-9 release from tumour infiltrating neutrophils in turn mediates release of VEGF from the ECM by HSPG cleavage. Then, soluble $VEGF_{165}$ diffuses to endothelial cells, initiating the angiogenic switch. Our study also indicates the value of human model systems, to confirm or supplement data obtained from mouse models and human tissues.

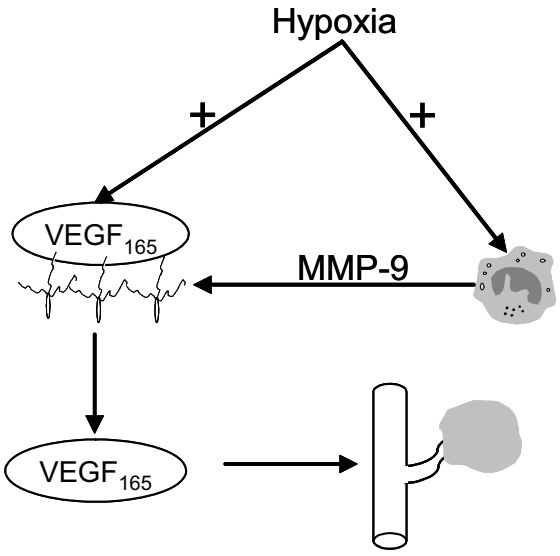

Figure 8. Proposed model for the role of neutrophil-derived MMP-9 in HSPG cleavage initiating the angiogenic switch by releasing VEGF in colorectal carcinomas.

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Conflict of interest statement

All authors declared no conflicts of interest.

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