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Research Article

Activity of MMP-19 inhibits capillary-like formation due to processing of nidogen-1

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Abstract. Matrix metalloproteinase 19 (MMP-19) is able to process various proteins of the basement membrane. To investigate the impact of MMP-19 activity on endothelial cells in the context of tumor extracellular matrix (ECM), we treated Matrigel matrix with an active recombinant MMP-19 and analyzed its effect on capillary-like formation. Human microvascular endothelial cells (HMEC-1) could not form capillary-like formation on Matrigel treated with recombinant MMP-19. Analyzing the Matrigel proteins, we found that MMP-19 preferentially cleaved nidogen-1. The cleavage site of nidogen-1 was mapped to Thr867-Leu868. This cleavage separates the G3 globular domain containing the binding site for the γ1 chain of laminin-1 and collagen IV and thus abolishes the capacity of nidogen-1 to cross-link ECM proteins. Anti-nidogen antibodies directed against the G3 domain of nidogen-1 inhibited the capillary-like structure formation to a similar extent as MMP-19. Since nidogen-1 is thought to stabilize microvessels, MMP-19 might be one of the enzymes that interferes with stabilization or maturation of nascent vasculature.

Key words. Extracellular matrix; matrix metalloproteinase; angiogenesis.

We have previously reported on a differential expression pattern of matrix metalloproteinase-19 (MMP-19) in blood-vessel-derived cells. MMP-19 is expressed by microvascular endothelial cells in vitro as well as by capillaries in the inflamed synovial membrane, whereas its expression is absent in quiescent macrovascular endothelial cells of large arteries and veins [1, 2]. MMP-19 was also detected in capillaries of normal mammary tissue, benign mammary carcinomas and in skin capillaries [3, 4]. MMP-19 was reported to have the capacity to degrade several basement membrane proteins such as type IV collagen, laminin, tenascin C, and nidogen [5]. This capacity together with the expression pattern may point to a pro-angiogenic function of MMP-19.

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MMPs, zinc-dependent endoproteinases that are able to degrade nearly all components of the extracellular matrix (ECM), play an essential role during the angiogenic process [6, 7]. They can directly influence angiogenesis by degradation of the basal lamina underlying the endothelium. This proteolysis enables outgrowth of sprouts and newly established blood vessels. Thus MT1-MMP, MMP-2, and MMP-9 that are produced by endothelial cells and whose activity is confined to the pericellular area are of great importance for the outgrowing process [8, 9]. Extracellular proteases can also influence the building of new blood vessels indirectly by release of pro-angiogenic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) from their ECM stores [10, 11]. Apart from these pro-angiogenic effects, MMPs also exhibit anti-angiogenic ef-

fects. They can for instance generate endostatin from collagen type XVIII [12, 13] and angiostatin from plasminogen [14–16]. Both these fragments are potent inhibitors of angiogenesis.

In the present study, we characterized the impact of MMP-19 on endothelial cells during angiogenesis using the tube-like structure formation assay on the tumor-derived matrix Matrigel. We show that a human microvascular endothelial cell line (HMEC-1) could not build the capillary-like structures on Matrigel treated with recombinant MMP-19 (rMMP-19). Using a degradomic approach, i.e. combination of two-dimensional (2D)-electrophoresis with mass spectrometry, we could identify nidogen-1 as the major substrate that was efficiently cleaved in the Matrigel treated with rMMP-19. Moreover, an anti-nidogen antibody was able to block the formation of capillary-like structures to a similar extent as the active rMMP-19. Finally, the cleavage site of nidogen-1 cross-linked in the solidified matrix was identified.

Based on our results, we propose that MMP-19 can interfere with the stabilization of nascent blood vessels because processing of nidogen-1 destabilizes the protein scaffold needed for this process.

Materials and methods

Cell culture and tube-like formation assay

HMEC-1 cells, kindly provided by Prof. Marmé (Freiburg, Germany), were cultured in endothelial cell growth medium MV (EGM-MV) (Promocell, Heidelberg, Germany) in a humidified atmosphere of 5% CO_2. The tube-like formation assay was adapted from Kubota et al. [17] and Donovan et al. [18]. For this purpose, frozen Matrigel was thawed on ice and an 80-µl aliquot was poured into each well of a microtiter plate. The plate was incubated for 30 min at 37 °C to allow the Matrigel to gel. After washing with EGM-MV, 2×10^4 HMEC-1 cells in 50 µl EGM-MV were seeded onto the matrix. Images were captured using a C3040 digital camera and a stereomicroscope SZ-ET (Olympus, Hamburg, Germany). Capillary-like formation, i.e. tubuli length, was evaluated after 20 h of incubation at 37 °C. Three independent experiments were conducted and at least three viewing fields evaluated for each experiment. The total tubuli length of each field was measured in pixels using the image analysis software Analysis (Soft Imaging Systems, Münster, Germany). The tubuli length is given as mean percentage of the control (± SD).

To study the effect of rMMP-19, Matrigel was incubated for 15 h with 600 U of the enzyme (75 pg/µl) in 50 µl TNC buffer [50 mM Tris-HCl pH 7.0, 0.15 mM NaCl, 10 mM $CaCl_2$, 0.05% (w/v) Brij 35, 20 µM $ZnCl_2$]. Either TNC buffer or rMMP-19 with the activity of only 60 U were used as a control (for definition of units see below).

To study the role of nidogen-1, 1.5 µg antibody specific for the G3 domain of nidogen-1 (clone JF2; Chemicon, Temecula, Calif.) were mixed with 80 µl liquid Matrigel. The Matrigel was incubated at 0 °C for 1 h and then gelled at 37 °C for 30 min before an experiment started. Rat isotype IgG was used as a control (Sigma, Seelze, Germany). Each assay was repeated three times.

Expression of *r*MMP-19 and determination of its activity

MMP-19 was produced as a fusion protein with glutathione-S-transferase (GST). MMP-19 protein ended with Arg, the first amino acid of the 36-amino-acid-long C-terminal tail. This C-terminal truncation improved the solubility of the protein. The enzyme was activated by incubating at 37 °C for 8 h after its purification using glutathione sepharose beads (Amersham, Freiburg, Germany) [19]. The proteolytic activity was monitored with the synthetic fluorogenic peptide substrate Mca-PLA-Nva-AR-NH_2 (Calbiochem, San Diego, Calif.) using a fluorometer F-2500 (Hitachi, Tokyo, Japan). For measurement, the fluorogenic peptide (2 µM) in 700 µl TNC buffer was mixed with 5 µl of rMMP-19. The fluorescence was excitated at 325 nm and monitored at 393 nm. The proteolytic activity was specified in units. One unit of MMP-19 activity was defined as an increase of one fluorescence unit per min under the in the experimental conditions described above. The reaction was performed at 22 °C and the concentration of the fluorogenic peptide was 2 µM.

Degradomics approach for identification of protease substrates

To identify proteins that were processed by MMP-19 in Matrigel (BD Biosciences, San Jose, USA), a two-dimensional (2D)-PAGE was used in combination with peptide mass fingerprinting. For the peptide mass fingerprinting, we used a modification of the Shevchenko et al. [20] protocol. Briefly, 20 µl gelled, growth-factor-reduced Matrigel was incubated at 37 °C for 16 h with 600 U of rMMP-19 in 30 µl TNC buffer. As a control, Matrigel was incubated in TNC buffer without MMP-19. Proteins were dissolved in 8 M urea, 2% CHAPS, 1% dithiothreitol, 0.002% bromphenol blue, and 0.5% Zoom Carrier Ampholytes (Invitrogen, Karlsruhe, Germany). A 20-µg aliquot of the protein mix was separated by isoelectric focussing using the Zoom Strips pH 4–7 (Invitrogen) and Zoom IPGRunner System (Invitrogen). NuPAGE Novex 4–12% Bis-Tris Zoom gels in the MES buffer (Invitrogen) were used for the second dimension. After the excision of a spot and its treatment with acetonitrile (ACN), the piece of gel was dried in a SpeedVac Plus (Thermo Savant, Holbrook, USA) and then incubated with 10 mM DTT and 100 mM NH_4HCO_3 followed by incubation with 55 mM iodoacetamide and 100 mM NH_4HCO_3 for 30 min. After sequential treatment with ACN and NH_4HCO_3, gel pieces were dried in a Speed-

Vac and then digested with trypsin (proteomics grade; Sigma) The gel pieces were extracted with 50 µl 0.1% trifluoroacetic acid (TFA) and 50% ACN, dried, and the peptides resuspended in 10 µl 0,1% TFA. Analysis was performed by the dried-droplet method according to Gobom et al. [21]. The mass spectra were recorded using a Bruker Biflex III (MALDI-TOF-MS) in the reflector mode. The proteins were identified by their cleavage pattern using the program ms-fit [22]. The results of identification were confirmed by immunoblotting using rat anti-nidogen-G3 antibodies (Chemicon).

Nidogen purification, cleavage and proliferation

The 150-kDa form of nidogen was purified from Matrigel according to a modified protocol of Paulsson et al. [23]. Briefly, a 400-µl aliquot of Matrigel was dissolved in 5 ml of 7 M urea, 0.05 M Tris/HCl pH 8.6, 2.5 M EDTA and then sequentially purified on the HiTrap-Q-FF and HiTrap-SP-FF column (Amersham, Freiburg, Germany) employing the ÄKTA-FPLC device (Amersham). A 25-µg aliquot of purified nidogen was incubated in TNC buffer for 16 h at 37 °C with rMMP-19. The processing was monitored by SDS-PAGE and immunoblotting using antiserum against nidogen-1 (kindly provided by Dr. Sasaki, MPI Biochemie, Germany). For proliferation tests, 15,000 HMEC-1 cells in 100 µl EGM-MV (Promocell) were incubated for 72 h at 37 °C in 96-well plates coated with 8 µg/cm^2 nidogen, 8 µg/cm^2 of MMP-19-processed nidogen and with rMMP-19 alone. Incorporation of ^{3}H-thymidine (Amersham) was measured using a scintillation counter Microbeta 1450 (Perkin-Elmer Wallac, Freiburg, Germany). Tests were done in triplicate.

Determination of cleavage site

A 30-µl aliquot of gelled Matrigel was incubated for 72 h at 37 °C with 200 U of rMMP-19 in TNC buffer. As a control, Matrigel was incubated without rMMP-19. The samples were analyzed using a NuPAGE Novex 4–12% Bis-Tris Zoom gel (Invitrogen).

For peptide mass fingerprinting analysis, the protein bands of interest were excised and analyzed as described above. For N-terminal sequencing, proteins were transferred to a PVDF membrane using 10 mM CAPS, 10% methanol, pH 11.0. The protein band of interest was excised and the N-terminal sequencing was carried out using a Procise Protein Sequencing System (Applied Biosystems, Foster City, USA).

Results

Capillary-like structure formation is inhibited by MMP-19 activity

Although microvascular endothelial cells express MMP-19 under various conditions, smooth muscle cells exhibit ongoing expression of this protein. Under inflammatory conditions or within tumors, even more cellular sources of MMP-19, apart from endothelial cells, come into play. Thus, the endothelium might be exposed to high concentrations of this enzyme. We therefore analyzed whether MMP-19 has an impact on the capacity of endothelial cells to build capillary-like structures.

HMEC-1 cells were seeded on solidified Matrigel matrix and the extent of tube-like structure formation was determined after 20 h incubation. To assess an effect of MMP-19, which we assumed would most likely consist in the proteolytic cleavage of tumor matrix, the solidified Matrigel was preincubated with rMMP-19 (600 units) before seeding of HMEC-1 cells. This preincubation resulted in a marked decrease of tube-like structures (fig. 1A). The mean tubuli length per viewing field was reduced to only 20 ± 7% of the untreated control (fig. 1B, D). Since the capillary formation was not affected by rMMP-19 exhibiting only weak activity, i.e., 60 units (fig. 1C), we could confirm that the activity of MMP-19 is responsible for this effect and that other factors that might be present in the enzyme preparation such as bacterial endotoxins were not involved in this inhibition. The amount of MMP-19 used for the experiments was comparable.

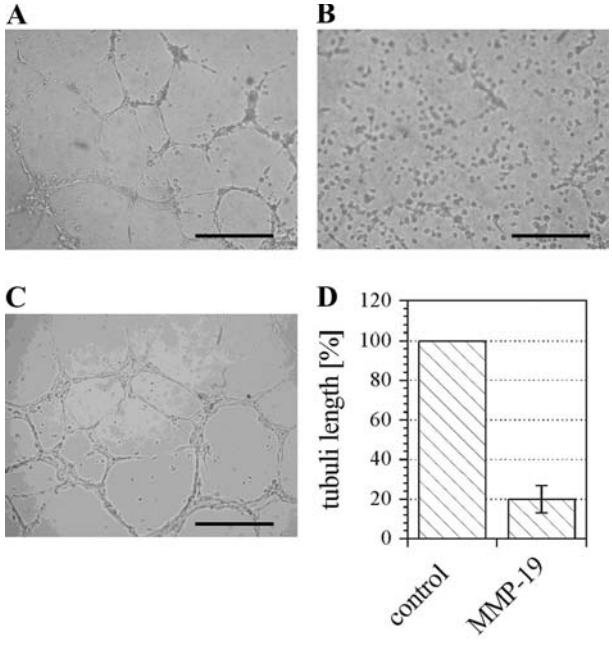

Figure 1. MMP-19 inhibits capillary-like formation. (*A*–*C*) The formation of the capillary-like structures was almost abrogated after the treatment of Matrigel with rMMP-19 exhibiting activity of 600 U (*B*), although HMEC-1 cells build these capillary-like structures on the untreated Matrigel within 20 h (*A*). Capillary formation was not affected by rMMP-19 exhibiting only weak activity, i.e., 60 U (*C*). Bar, 50 µm. (*D*) Quantification of the tube-like structure formation. The length of tubuli-like structures was measured in at least three viewing fields (see Materials and methods). The mean ± SD of the tubuli length per field of three independent experiments is given as the percentage of the control.

Nidogen, an abundant component of tumor matrix, is cleaved by MMP-19

In an effort to elucidate which molecules are degraded by MMP-19 and are thus responsible for the observed inhibition of capillary-like formation, we studied cleavage of Matrigel proteins using a proteomic approach. The Matrigel matrix either untreated or treated with rMMP-19 was analyzed using 2D-PAGE. Matrigel was incubated in the solid state so that the protein-protein interactions between its components were established. Although many proteins showed smeared bands due to multiple modifications of the ECM proteins, a number of abundant protein spots could be clearly recognized. Only one newly generated, intense spot, with a pI of 5.7 and molecular mass of 66 kDa, was identified in the rMMP-19-treated sample (fig. 2A). This spot was excised, treated with trypsin, analyzed by peptide mass fingerprinting and identified using the ms-fit program as nidogen-1 (score 3.4×10^8). Another spot containing nidogen-1 was identified at 105 kDa (spot 2). The 150-kDa form of nidogen could not be found in this gel. Since we could not identify additional fragments of nidogen in the 2D-PAGE analysis, we further analyzed its processing using immunoblotting with an antibody against the C-terminal part of nidogen-1 (fig. 2B). This antibody identified two bands of 105 and 40 kDa. The 105-kDa band corresponded with spot 2 in the 2D-PAGE. The 40-kDa band was not seen on the 2D-PAGE and represented the C-terminal fragment of nidogen-1. Thus, this fragment did not appear in the 2D-PAGE analysis not because of its further degradation by MMP-19 but due to the particular conditions used for protein solubilisation and isoelectric focusing. Most likely, this fragment precipitated during isoelectric focusing due to insufficient solubilization in the urea buffer used. Thus, although the fragment was detected by immunobloting, silver staining was not sensitive enough to detect it. The 66-kDa fragment detected in the 2D-PAGE analysis could represent the N-terminal fragment of the 105-kDa form of nidogen-1. The 150-kDa form of nidogen was probably not efficiently extracted or resolved and therefore we could detect it by 2D-PAGE or immunoblotting. Thus, using the proteomics approach, we could identify nidogen-1 as a new substrate of MMP-19 that was processed in the solid Matrigel matrix.

Antibody against the G3 domain of nidogen inhibits capillary-like formation

From the above experiment we assumed that MMP-19 inhibits capillary-like structure formation by cleaving nidogen-1. To confirm the role of nidogen-1 independently of MMP-19 activity, we used the anti-nidogen antibody specific for the C-terminal G3 domain to inhibit the capacity of nidogen to cross-link the ECM proteins. The antibody was incubated with liquid Matrigel for 1 h before its solidification. As a control, the same amount of rat IgG was applied to Matrigel. Whereas HMEC-1 cells grown on Matrigel supplemented with the control antibody were able to build the capillary-like structure, their capacity was considerably inhibited by the anti-nidogen (anti-G3 domain) antibody (fig. 3A, B). Quantification of three independent experiments showed that the formation of capillary structures dropped to 33 ± 6% of the control (fig. 3C).

This experiment confirms the role of nidogen in the formation of capillaries and further supports our finding that MMP-19 activity influences the capillary formation process.

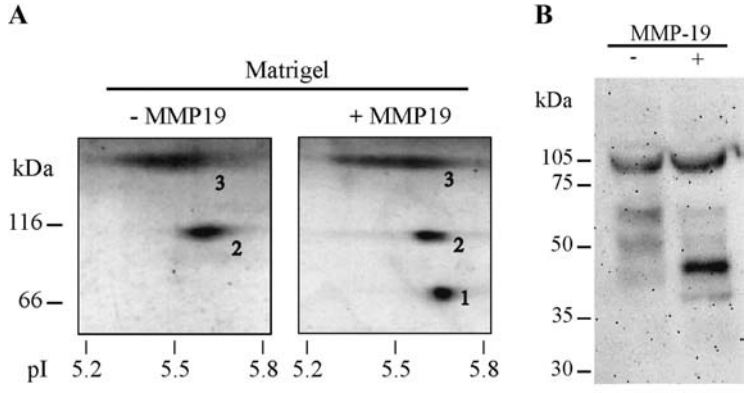

Figure 2. MMP-19 processes nidogen-1 in the tumor matrix. The solid Matrigel was incubated with rMMP-19 or with the reaction buffer as a control (*A*). The proteins were separated using 2D-PAGE and visualized via silver staining. After the rMMP-19 treatment, a novel protein spot (1) emerged at 66 kDa and was identified as a fragment of nidogen-1 by peptide mass fingerprinting. The spot (2) was identified analogously as nidogen-1. Additional fragments of nidogen-1 could not be found by the 2D-PAGE analysis. Spot (3) was identified as the γ1 chain of laminin-1. Antibody against the G3 domain of nidogen-1 was used to identify potential cleavage products of the C-terminal part of nidogen-1 (*B*). A 40-kDa fragment containing the G3 domain of nidogen-1 with binding sites for type IV collagen and laminin 1 was identified as the only C-terminal product.

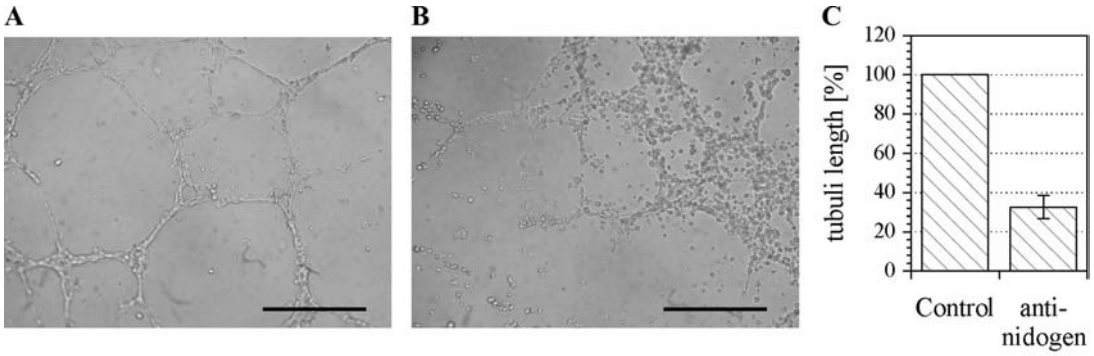

Figure 3. Blocking of nidogen-1 by antibodies against the G3 domain inhibits capillary-like formation. Capillary-like formation was compared between HMEC-1 cells grown on Matrigel containing antibody against the G3 domain of nidogen-1 (*B*) and IgG1 isotype control (*A*). While the control antibody did not affect the capillary-like formation, the anti-nidogen antibody reduced the mean tubuli length by 67% after 20 h of cell culture (*C*). The length of tubuli-like structures was measured in at least three viewing fields. The mean ± SD of the tubuli length per field of three independent experiments is given as a percentage of the control. Bar, 50 µm.

Processing of nidogen-1 does not influence the proliferation of endothelial cells

Since the cleavage of nidogen-1 could also result in cellular effects other than the inhibition of capillary-like structure formation, we tested whether proliferation of HMEC-1 cells was also affected. Nidogen-1 was purified from Matrigel using a combination of anion and cation exchange chromatography and then processed with rMMP-19. MMP-19 generated two predominant nidogen fragments with molecular masses of 66 and 40 kDa (fig. 4A). These fragments most likely corresponded to those found after the treatment of Matrigel with rMMP-19. However, the higher-molecular-mass forms of nidogen-1 also appeared to be processed by rMMP-19. Nevertheless, no other fragments, except those of 66 and 40 kDa, could be identified.

Both the purified uncleaved and purified processed nidogen-1 were used to coat culture plates and the proliferation of HMEC-1 was assayed using a ^{3}H-thymidine incorporation assay (fig. 4B). Since remnants of rMMP-19 were present in the sample with cleaved nidogen-1, plates coated with rMMP-19 or uncoated plates were used as a control. HMEC-1 showed comparable proliferation rates on all plates.

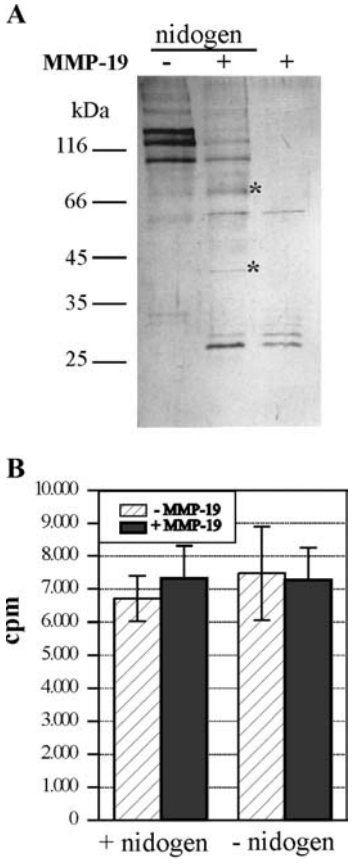

Figure 4. Processing of nidogen does not change the proliferation rate of HMEC-1. Nidogen-1, purified from Matrigel was processed with rMMP-19 (*A*). A silver-stained polyacrylamide gel shows enriched nidogen-1 (–), cleavage of nidogen-1 by rMMP-19 (+) and the rMMP-19 preparation without nidogen-1. Asterisks denote fragments of nidogen-1. Cleavage of nidogen-1 did not influence the proliferation of HMEC-1 (*B*). Proliferation of HMEC-1 cells was performed on cell culture vessels coated with either purified nidogen-1 or purified, cleaved nidogen-1. Proliferation was assayed by incorporation of ^{3}H-thymidine and the proliferation rate is given in counts per minute (cpm) with the SD indicated by error bars.

MMP-19 cleaves off the G3 domain of nidogen

To further elucidate the mechanism of inhibition of capillary-like structure formation due to processing of nidogen-1, the exact cleavage site was determined.

Incubation of solidified Matrigel with rMMP-19 (600 U) resulted in processing of nidogen-1 into 66-kDa and 40-kDa proteolytic fragments (fig. 5A). The band seen at 30 kDa was identified as a fragment of GST-MMP-19 (glutathione-S-transferase) that was present in all preparations of rMMP-19. Using a peptide mass fingerprinting analysis, the 66-kDa band was identified as an N-terminal fragment of the 105-kDa form of nidogen-1 and the 40-kDa band as its C-terminal fragment. Furthermore, in

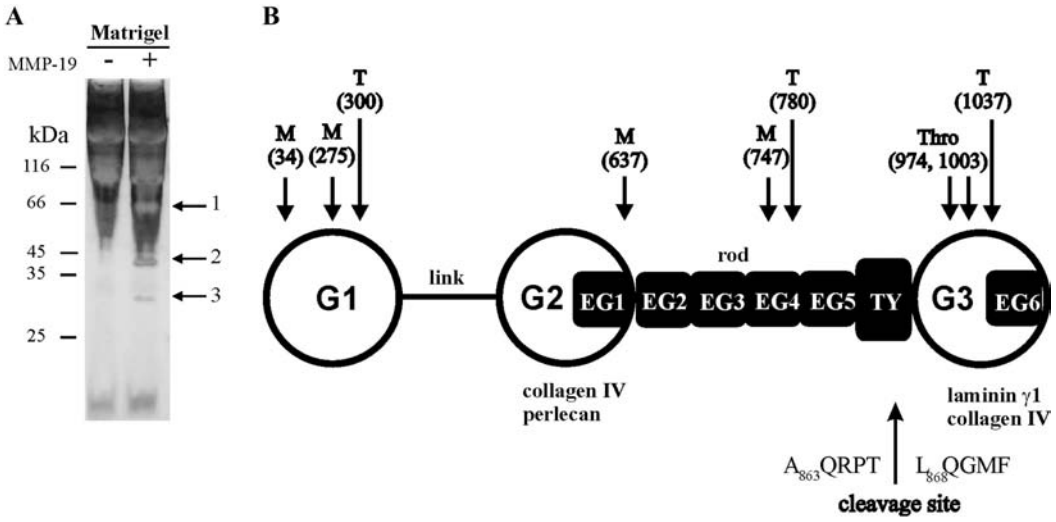

Figure 5. Identification of the cleavage site of MMP-19 in nidogen-1 and comparison of the known cleavage sites. Solidified Matrigel was incubated with rMMP-19 (+) or with the reaction buffer as a control (–). Three bands appeared after the incubation with rMMP-19 (arrows) (*A*). The 66-kDa-band (1) was identified by peptide mass fingerprinting as the N-terminal fragment of the 105-kDa form of nidogen-1. The 40-kDa band (2) was identified as the C-terminal fragment, and the 30-kDa band (3) as a GST-MMP-19 fragment. This fragment was present in the MMP-19 preparation. Band 2 was further analyzed by N-terminal sequencing. (*B*) diagram of nidogen-1 with cleavage sites. Binding sites of ECM components are shown below the domains. Cleavage sites with known cleavage positions of other proteases are indicated at the top. M, matrilysin (MMP-7); T, trypsin; Thro, thrombin [40, 41]. EG, EGF-like domain; TY, thyreoglobulin-like domain The diagram was adapted from Ries et al. [25].

the N-terminal fragment, we could identify fragments between positions 318 and 808 and in the C-terminal fragments between positions 969 and 1189. The cleavage site was thus narrowed down between positions 808 and 969. For further refinement of the cleavage site, the N terminus of the 40-kDa C-terminal fragment was sequenced. The N-terminal sequence of the C-terminal fragment was identified as NH_2-L-Q-X-(M)-F where X stands for not defined and (M) for ambiguously identified. Thus, the cleavage site lies between Thr^{867} and Leu^{868} within the stretch NH_2-AQRP**T/L**QGMFP. This position is localized N-terminally of the thyreoglobulin domain which starts at position 870 of nidgogen-1. Since the G3 domain contains binding sites for type IV collagen and laminin γ1, this cleavage separates the G3 domain from the N-terminal part of nidogen-1 (fig. 5B), thereby impairing the cross-linking capacity of nidogen-1 [24, 25].

Discussion

MMPs are able to degrade many proteins of the basement membrane and interstitial tissue. This facilitates endothelial cell growth out of the established vascular bed to build new blood vessels. Studies involving MMP-deficient mice have shown the importance of MMPs during angiogenesis. MMP-2-deficient mice exhibit partial inhibition of angiogenesis and MMP-9-deficient mice show a diminished growth plate angiogenesis [26–28]. MT1-MMP seems also to be important for the neovascularization process as invasion-incompetent cells expressing a fibrinolytically active, transmembrane-deleted form of MT1-MMP remain non-invasive [29]. Although numerous publications show the importance of MMPs for angiogenesis, much less is known about the role of MMPs during the maturation and stabilization of blood vessels, i.e., about their functions during the late stages of angiogenesis or during blood vessel regression. The maturation of blood vessels is not only characterized by the stabilization of vessels by pericytes and smooth muscle cells but also by laying down of ECM proteins that create the new basement membrane [30, 31].

Investigating the impact of MMP-19 on endothelial cells we showed in this study that cleavage of nidogen-1 by MMP-19 leads to inhibition of capillary-like structure formation.

We have previously shown that MMP-19 is expressed in certain capillary endothelial cells co-expressing vascular endothelial growth factor receptor 2 (VEGF-R2, KDR) and integrin $\alpha v\beta 3$ but not TIMP-1 [1]. Capillaries and small blood vessels found in psoriatic lesions and other proliferative cutaneous diseases also showed positive reactivity for the MMP-19 antibodies [32]. Moreover, smooth muscle and endothelial layers of all blood vessels in breast tumors such as fibrocystic disease and in fibroadenomas were intensely stained with anti-MMP-19 antibodies. However, MMP-19 was evidently absent from all vessels within the neighborhood of ductal and lobular carcinomas that represent an invasive cancer form [3].

According to our previous results, endothelium of mature blood vessels is surrounded by mural cells exhibiting constitutive and strong expression of MMP-19. Within breast tumors also, endothelial cells are enclosed among tumor cells producing MMP-19 [3, 4, 32]. What is the consequence of MMP-19 activity for the nascent blood vessels in such an environment?

To characterize the role of MMP-19 during angiogenesis, we studied capillary-like structure formation in vitro. This morphogenesis assay was carried out on ECM extracted from fibrosarcoma tumors (Matrigel) that was incubated with rMMP-19. Surprisingly, the building of capillary-like structures by HMEC-1 cells on the matrix treated by MMP-19 was considerably inhibited.

To gain further insight into the mechanism of inhibition of in vitro angiogenesis by MMP-19, we analyzed the Matrigel matrix using 2D-PAGE. The treatment of Matrigel with rMMP-19 generated an additional protein spot of 66 kDa that was identified by peptide mass fingerprinting as nidogen-1.

Nidogen-1 is an abundant component of the basement membrane and the tumor matrix. It is composed of three globular regions and a rigid stalk that together generate an asymmetric dumbbell-like structure [33, 34] (fig. 5B). The G2 domain binds to collagen IV and heparan sulfate, and the G3 domain binds to collagen IV and the γ1 chain of laminin [24, 25]. Although nidogen has been hypothesized to function as a link between collagen IV and laminin in the basement membrane [33], the function of nidogen-1 in the course of various angiogenesis steps has not been fully established. Sprouting of capillary-like structures in an aortic explant model was shown to be dependent on the concentration of a laminin-nidogen complex and nidogen is expressed during the entire angiogenic process in a wound-healing model [35, 36]. The relevance of the interaction between nidogen and laminin was shown during branching morphogenesis of the submandibular gland and during lung and kidney development [37, 38]. An inhibitory effect was also seen during involution of mammary glands that was induced by cleavage of nidogen by MMP-3 [39].

To support the hypothesis that cleavage of nidogen inhibits capillary-like structure formation, we embedded antibodies directed against the G3 domain of nidogen within the Matrigel matrix. This treatment resulted in abrogation of capillary-like structure formation presumably via blockade of the binding site for the γ1 chain of laminin. This effect resembles the inhibition of branching morphogenesis of the submandibular gland by antibodies against the nidogen-binding epitope of the laminin γ1 chain [37]. Furthermore, analyzing the processing of nidogen-1 by MMP-19, we found that the cleavage site is located immediately before the thyreoglobulin type II domain, which starts at position 870. By cleaving the peptide bond between T^{867} and L^{868}, the 105-kDa form of nidogen-1 that is found in Matrigel, is cleaved to the 66-kDa fragment and the C-terminal 40-kDa fragment (fig. 5B). The 150-kDa form of unprocessed nidogen-1 could not be identified in 2D-PAGE analysis; it might be N-terminally processed in the Matrigel matrix, although no corresponding cleavage fragment was identified. Such a cleavage could be due to 'endoproteolytic processing' of nidogen-1 as described by Paulsson et al. [23].

Although nidogen was identified previously as a substrate of MMP-19 by incubation of the catalytic domain of MMP-19 with purified nidogen in vitro [5], we showed here that the full-length MMP-19 expressed as a GST-fusion protein is able to cleave nidogen-1 in the complexity of a cross-linked matrix. Nidogen has also been reported to be processed by other MMPs and serine proteases [40, 41]. Thus, matrilysin (MMP-7) exhibits high proteolytic activity toward nidogen, while MMP-1 and MMP-2 show only weak activity [41]. Comparison of the nidogen proteases and their cleavage sites is shown in figure 5.

The cellular effects of the nidogen processing could be mediated either directly by destruction of the binding epitopes of integrins or indirectly by structural alterations of the ECM. Since the known binding sites for integrins are located in the epidermal growth factor (EGF) repeat of the G2 domain and in the RGD motif in the rod region between G2 and G3 around position 700, they cannot be directly affected by the MMP-19 activity. Processing of nidogen-1 by MMP-19 did not change the proliferation rate of endothelial cells in comparison to the unprocessed protein. Therefore, we suppose that removing the G3 domain from nidogen-1 disrupts its ability to cross-link the collagen IV and laminin network which in turn results in structural alterations of ECM by destabilization of the protein scaffold necessary to support morphogenesis of capillaries. This capacity of MMP-19 to impede formation of capillary structures could have extensive consequences for tumor growth.

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