The NC1 domain of type XIX collagen inhibits *in vivo* melanoma growth

Laurent Ramont,[1] Sylvie Brassart-Pasco,[1] Jessica Thevenard,[1] Aurélie Deshorgue,[1] Lydie Venteo,[2] Jean Yves Laronze,[3] Michel Pluot,[2] Jean-Claude Monboisse,[1] and François-Xavier Maquart[1]

[1]Laboratoire de Biochimie Médicale et Biologie Moléculaire Centre National de la Recherche Scientifique Unite Mixte de Recherche 6198 [2]Laboratoire d'Anatomie Pathologie, Faculté de Medecine; and [3]Centre National de la Recherche Scientifique; Formation de Recherche en Evolution (FRE) 2715: Isolation, Structure, Transformation et Synthèse de Substance Naturelles, Institut Fédératif de Recherche 53 Biomolécules, Université de Reims Champagne-Ardenne, Reims, France

Abstract

Type XIX collagen is a minor collagen that localizes to basement membrane zones, together with types IV, XV, and XVIII collagens. Because several NC1 COOH-terminal domains of other chains from basement membrane collagens were reported to exhibit antitumor activity, we decided to study the effects of the NC1(XIX) collagen domain on tumor progression using an experimental *in vivo* model of mouse melanoma. We observed a 70% reduction in tumor volume in NC1(XIX)-treated mice compared with the corresponding controls. Histologic examination of the tumors showed a strong decrease in tumor vascularization in treated mice. *In vitro*, NC1(XIX) inhibited the migrating capacity of tumor cells and their capacity to invade Matrigel. It also inhibited the capacity of human microvascular endothelial cells to form pseudotubes in Matrigel. This effect was accompanied by a strong inhibition of membrane type-1 matrix metalloproteinase (matrix metalloproteinase-14) and vascular endothelial growth factor expression. Collectively, our data indicate that the NC1 domain of type XIX collagen exerts antitumor activity. This effect is mediated by a strong inhibition of the invasive capacities of tumor cells and antiangiogenic effects. NC1(XIX) should now be considered as a new member of the basement membrane collagen-derived matrikine family with antitumor and antiangiogenic activity. [Mol Cancer Ther 2007;6(2):506–14]

Introduction

Type XIX collagen is a minor collagen initially isolated from human rhabdomyosarcoma libraries (1, 2). It belongs to the fibril-associated collagens with interrupted triple helix collagen family (3). It is localized in the basement membrane zone of vascular, neuronal, mesenchymal, and epithelial tissues, associated with types IV, XV, and XVIII collagens (4, 5). The α1(XIX) chain is constituted of a 268-residue NH_2-terminal domain, an 858-residue discontinuous collagenous domain, and a short 19-residue COOH-terminal noncollagenous NC1 domain. It seems to be involved in muscle differentiation, esophageal muscle physiology, and morphogenesis, but other functions cannot be excluded (6, 7).

COOH-terminal NC1 domains of other basement membrane collagen chains were reported to exhibit antitumor activity and antiangiogenic properties. For instance, endostatin, the NC1 domain of α1(XVIII), restin, the NC1 domain of α1(XV), tumstatin, the NC1 domain of α3(IV), canstatin, the NC1 domain of α2(IV), and arresten, the NC1 domain of α1(IV) collagen chains were shown to control tumor development and/or angiogenesis in several experimental models (for review, see ref. 8).

Angiogenesis constitutes a crucial step for the development of solid tumors (9). The new vessels bring the nutriments necessary to tumor development. Tumor angiogenesis depends on an imbalance between proangiogenic and antiangiogenic factors (10, 11). In addition, peptides generated by partial proteolysis of extracellular matrix macromolecules, also called matrikines or matricryptines, may be implied in this control (12, 13).

Extracellular matrix remodeling is essential for tumor cell migration and angiogenesis in tumor progression. Matrix metalloproteinases (MMP) are the main enzymes implicated in this process (14). Membrane type-1 MMP (MT1-MMP or MMP-14), a membrane-anchored protease expressed by tumor and endothelial cells, plays a crucial role in cell migration (15). MT1-MMP not only degrades extracellular matrix but also controls the activation of other MMPs, particularly pro-MMP-2 (16, 17). MT1-MMP promotes tumor invasion and growth (18, 19). Moreover, this enzyme controls the migration of endothelial cells and promotes angiogenesis during tumor development (20, 21). Recently, MT1-MMP was shown to be involved in the control of neovascularization in physiologic conditions or in tumor

Received 4/14/06; revised 11/14/06; accepted 12/21/06.

Grant support: Université de Reims-Champagne-Ardenne, the Centre National de la Recherche Scientifique, the Ligue Nationale contre le Cancer (Comité de la Haute Marne), the Association pour la Recherche sur le Cancer, and the Cancéropole Grand Est.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Laurent Ramont, Laboratoire de Biochimie Médicale et Biologie Moléculaire, Faculté de Medecine, Centre National de la Recherche Scientifique Unité Mixte de Recherche 6198, Institut Fédératif de Recherche 53 Biomolecules, Université de Reims Champagne-Ardenne, 51 rue Cognacq Jay, F-51095 Reims Cedex, France.
Phone: 33-3-26-78-31-81; Fax: 33-3-26-78-85-39.
E-mail: lramont@chu-reims.fr

doi:10.1158/1535-7163.MCT-06-0207

invasion, independently of pro-MMP-2 activation (22). Overexpression of MT1-MMP was associated with an increase in *in vivo* tumor growth and vascularization in a melanoma model (23).

In this article, we decided to study the effects of the NC1(XIX) domain on tumor progression and angiogenesis in a mouse melanoma model. We showed that NC1(XIX) domain decreased *in vivo* tumor growth through an inhibition of melanoma cell invasive properties and tumor angiogenesis.

Materials and Methods

Reagents

Culture medium and molecular biology products were from Life Technologies (Invitrogen, Cergy Pontoise, France). Bovine serum albumin (BSA) and Matrigel (ECM gel) were purchased from Sigma (St. Quentin Fallavier, France). Rat anti-mouse CD31 was from PharMingen (distributed by BD Biosciences, Le Pont de Claix, France). Goat anti-mouse MT1-MMP, goat anti-mouse vascular endothelial growth factor (VEGF), and goat anti-mouse actin (I-19) were from Santa Cruz Biotechnology (distributed by Tebu, Le Perray-en-Yvelines, France). Rabbit anti-human MT1-MMP was from Chemicon (distributed by Euromedex, Souffelweyersheim, France).

Cell Cultures

B16F1 cells, a lung metastatic subline of murine B16 melanoma, were a generous gift from Dr. M. Grégoire (Institut National de la Sante et de la Recherche Medicale Unité Mixte de Recherche Scientifique 419, Nantes, France). They were grown in RPMI 1640 supplemented with 5% fetal bovine serum in 25-cm^2 flasks (Nunclon, Dutscher, Brumath, France) at 37°C in a humid atmosphere (5% CO_2, 95% air).

Human microvascular endothelial cells-1 (HMEC-1) were obtained from E.W. Ades (Center for Disease Control and Prevention, Atlanta, GA). They were cultured in endothelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with 0.4% (w/v) endothelial cell growth supplement/heparin, 5% (v/v) FCS, 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone, and 50 ng/mL amphotericin B.

Peptides

The NC1(XIX) peptide, NPEDCLYPVSHAHQRTGGN, and the corresponding scrambled peptide, AGNEQP-NYHSDPGTHLCRV, were obtained by solid-phase synthesis using an *N*-(9-fluorenyl)methoxycarbonyl derivative procedure. They were further purified by reverse-phase high-performance liquid chromatography with a C18 column eluted by a gradient of acetonitrile in trifluoroacetic acid and then lyophilized (24).

Animals

C57Bl6 mice (average body weight, 16–18 g) were purchased from Harlan France (Gannat, France). Animals were individually caged and given food and water *ad libitum*. They were kept in a room with constant temperature and humidity. All mice were acclimatized to our laboratory conditions for 1 week before starting the experiments. The *in vivo* experiments were conducted according to the ethical guidelines of the Centre National de la Recherche Scientifique.

In vivo Tumor Growth Measurement

A suspension of B16F1 cells (2.5×10^5 in 0.1 mL RPMI 1640) was s.c. injected into the left side of syngeneic C57Bl6 mice. Four types of experiments were done. In the first set of experiments, B16F1 cells were preincubated for 15 min with either RPMI (control), NC1(XIX) peptide (30 μmol/L), or the corresponding scrambled peptide at the same concentration before the initial injection. Control medium, NC1(XIX) peptide (2.5 mg/kg mouse weight), or scrambled peptide (2.5 mg/kg mouse weight) was then reinjected around the tumor at days 7 and 14. In the second set of experiments, preincubation of cells with NC1(XIX) was done but peritumoral injections at days 7 and 14 were omitted. In the third set of experiments, preincubation of B16F1 cells with NC1(XIX) was omitted and only peritumoral injection at days 7 and 14 were done. Finally, in the fourth set of experiments, control medium, NC1 1(XIX) (10 mg/kg mouse weight), or scrambled peptide (10 mg/kg mouse weight) was injected i.p. at days 10 and 15 after appearance of the tumor. In all these experiments, the groups contained six or seven mice. Tumor volumes were determined according to $V = 1/2\ A \times B^2$, where A denotes the largest dimension of the tumor and B represents the smallest dimension (25). Tumor volume was measured on days 7, 14, 18, and 21. At day 21, mice were sacrificed and tumors were surgically extracted for morphologic and biochemical studies.

Tumor Extract Preparation

Tumors were surgically extracted at day 21 and homogenized in a Tris buffer [50 mmol/L Tris (pH 7.6), 0.5 mol/L NaCl, 0.1% SDS, 0.02% NaN_3, 1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L EDTA, 1 mmol/L iodoacetamide] and proteins were quantified by the Bradford method (26).

Morphologic Studies

Tissue sections were cut at 5 μm and stained with hematein-erythrosin-safran for routine histologic analysis and by Masson trichrome for evaluating angiogenesis.

Immunohistochemical analysis for VEGF and MT1-MMP was done on paraffin-embedded 3-μm tissue sections from tumor specimens. The sections were deparaffinized, rehydrated, and treated in citrate buffer (pH 6.0), for antigen retrieval. They were treated for 10 min with 3% H_2O_2 in PBS to block endogenous peroxidases. For CD31 labeling, frozen tumor specimens were cut in 5-μm sections using a cryostat apparatus, put on a coated glass slide, and fixed in acetone. Sections were preincubated for 20 min with goat serum and incubated overnight at 4°C with a rat anti-mouse CD31, goat anti-mouse MT1-MMP antibody, or goat anti-mouse VEGF antibody followed by biotinylated rabbit anti-goat or anti-rat, peroxidase-labeled streptavidin, the peroxidase substrate 3-amino-9-ethyl-carbazole (Vector Laboratories, Burlingame, CA), and H_2O_2, which gives a dark red reaction product. Tumor sections were counterstained with

hematoxylin. Semiquantitative evaluation of VEGF and MT1-MMP expression was done by two independent pathologists. At least three different fields were counted in six or seven different tumors per group [control, NC1(XIX), or scrambled peptide]. Expression was graded from 0 to 3 according to the patterns as follows: 0, no positivity; +, faint labeling; ++, strong labeling; and +++, intense labeling. The number of fields classified in each group was considered for analysis of the data.

Cell Proliferation

For measuring cell proliferation, 10,000 cells were plated into 96-well plates and incubated for 24, 48, or 72 h with NC1(XIX) peptides in RPMI 1640 supplemented with 5% fetal bovine serum for B16F1 cell or in endothelial cell growth medium for HMEC-1. Cell proliferation was measured with colorimetric assay WST-1 (Roche Diagnostics, Meylan, France).

Cell Adhesion

For measuring cell adhesion, two types of experiments were done. In the first set of experiments, non-tissue culture plates were coated with Matrigel. Fifty thousand B16F1 cells per well were seeded into 96-well plates and incubated for 30 min. NC1(XIX) peptide (30 μmol/L) was added to the culture medium during the adhesion assay. In the second set of experiments, NC1(XIX) peptide or scrambled peptide was immobilized on 96-well plates at different concentrations (200 ng, 1 μg, and 10 μg per well). Fifty thousand B16F1 cells per well were seeded and incubated for 30 min. In all experiments, plastic was used as a positive control and BSA (1 or 10 μg per well) was used as a negative control. After several rinses, adhered cells were measured with colorimetric assay WST-1.

Apoptosis

Apoptosis was determined by fluorescent microscopy using cell staining with Hoechst 33342. After 4 h of exposure to NC1(XIX) or scrambled peptide (30 μmol/L), cell morphologic changes typical of apoptosis, such as chromatin condensation or fragmentation and nuclear shrinkage, were studied.

In vitro Migration and Invasion Assays

Migration and invasion were assayed in Transwell devices (Costar, distributed by Dutscher, Brumath, France). RPMI 1640 supplemented with 10% fetal bovine serum and 2% BSA was used as a chemoattractant. Briefly, 40,000 cells were suspended in serum-free RPMI 1640 containing 0.2% BSA and seeded onto the Transwell membranes (6.5 mm in diameter and 8-μm pores) coated (invasion assay) or not (migration assay) with Matrigel (30 $\mu g/cm^2$). After 24 or 48 h of incubation period for migration or invasion studies, respectively, cells were fixed with methanol and stained with crystal violet for 15 min. Cells remaining on the upper face of the membranes were scrapped and those on the lower face were counted using an inverted microscope.

Capillary Tube Formation on Matrigel

Matrigel (10 mg/mL) was added to a 24-well culture plate (200 μL per well). After 30 min of incubation at 37°C, 50,000 HMEC-1 cells were suspended in serum-free endothelial cell growth medium and seeded onto the gel. They were incubated with either control medium, NC1(XIX), or the corresponding scrambled peptide (30 μmol/L). Capillary tube formation was observed after 48 h. Semiquantitative evaluation of the capillary tubes was done after pixelization by determining the ratio of black pixels relative to the total pixels. Quantitative evaluation of the capillary tubes was further done by a second method using computer analysis ImageJ software for Windows (NIH, Bethesda, MD; ref. 27).

MT1-MMP Expression and Quantification

Western blot analysis was used to evaluate MT1-MMP expression by HMEC-1 and B16F1 cells. For that purpose, 50 μg proteins from cell membrane extracts were deposited into the gels, subjected to SDS-PAGE, and then transferred onto Immobilon-P membranes. Membranes were blocked by incubation with 5% nonfat dry milk, 0.1% Tween 20 in 50 mmol/L Tris-HCl buffer, and 150 mmol/L NaCl (pH 7.5) for 2 h at room temperature. They were incubated overnight with a rabbit anti-human MT1-MMP antibody and then for 1 h with the corresponding peroxidase-conjugated second antibody at room temperature. Immune complexes were visualized using the enhanced chemiluminescence detection kit (Amersham, Orsay, France).

Quantification of MT1-MMP activity was done using the Biotrak MT1-MMP activity assay (Amersham) according to the manufacturer's instruction.

Western Blot Analysis of VEGF Expression *In vivo*

Western blot analysis was used to evaluate VEGF expression in tumor extract preparation. For that purpose, tumors were surgically collected, and tumor extracts were prepared as described above. Fifty micrograms of proteins from tumor extracts were deposited on the gels, subjected to SDS-PAGE, then transferred onto Immobilon-P membranes. Membranes were blocked and incubated overnight with goat anti-mouse VEGF antibody or actin antibody and then for 1 h with the corresponding peroxidase-conjugated second antibody at room temperature. Immune complexes were visualized with the enhanced chemiluminescence detection kit.

Real-time Reverse Transcription-PCR Analyses

RNA isolation was done using the Qiagen RNeasy kit (Courtaboeuf, France) according to the manufacturer's instructions. cDNA was prepared from 1 μg of total RNA by reverse transcription. FastStart DNA Master Hybridization kit (Roche Diagnostics) was used for the PCR. The Roche Diagnostics LightCycler was used for amplification and data collection. The primers were 5′-cgggtgaggaataaccaagt-3′ and 3′-ccagaagagagcagcatcaa-5′ for MT1-MMP; 5′-attcctatgtgggcgacgag-3′ and 3′-atggctggggtgttgaag-5′ for β-actin; and 5-′acgcatttggtcgtattggg-3′ and 3′-tgattttggagggatctcgc-5′ for glyceraldehyde-3-phosphate dehydrogenase. The denaturation step was 8 min at 94°C. The amplification step was 50 cycles of 95°C for 0 s, 60°C for 10 s, and 72°C for a time depending on the fragment length. Fluorescence acquisition was carried out at 72°C in single mode at the end of the elongation step. The fusion step was done at 65°C to 95°C (temperature transition 0.2°C/s) with stepwise fluorescence acquisition. Product specificity was

evaluated by melting curve analysis and by electrophoresis in 2% agarose gel. Fluorescence was analyzed by the LightCycler Data Analysis software (Roche Diagnostics). Crossing points were established using the second derivative method. Results were expressed as the target/internal standard (glyceraldehyde-3-phosphate dehydrogenase) concentration ratio of the sample divided by the target/internal standard concentration ratio of the calibrator.

Statistical Analyses

For *in vitro* experiments, statistical analyses were done by Student's *t* test and results expressed as mean ± SD. For *in vivo* experiments, volumes of primary tumors were statistically analyzed using the nonparametric *U* test of Mann and Whitney. Statistical analysis of the immunohistochemistry data was done using the χ^2 test.

Results

NC1(XIX) Inhibits *In vivo* Tumor Growth

S.c. injection of B16F1 melanoma cells in C57Bl6 mice resulted in the development of a tumor at the injection site. Preincubation of melanoma cells with NC1(XIX) (30 μmol/L) followed by peritumoral injections of the peptide (2.5 mg/kg mouse weight) at days 7 and 14 significantly decreased tumor growth (Fig. 1A). Tumor volume was significantly decreased as soon as day 14 compared with untreated (control) mice or with scrambled peptide–treated mice. The difference between control or scrambled-treated mice and NC1(XIX)-treated mice increased with time, so that tumor volume was decreased by 70% ($P < 0.01$) at day 21. Preincubation of melanoma cells with NC1(XIX) (30 μmol/L) without reinjections had no significant effect on tumor growth (Fig. 1B). When the preincubation of tumor cells with NC1(XIX) was omitted and only NC1(XIX) injections at days 7 and 14 were done, a strong inhibition of tumor growth was observed, nearly as intense as the full course treatment (−69%; $P < 0.01$; Fig. 1C). When the peptide was injected i.p. on days 10 and 15 after the appearance of the tumor, we also observed a strong reduction in tumor growth (−67%; $P < 0.01$) compared with the scrambled peptide or control medium (Fig. 1D).

NC1(XIX) Has No Effects on *In vitro* Melanoma Cell Growth and Cell Adhesion

To check if NC1(XIX) was able to inhibit *in vitro* melanoma cell growth, 10,000 B16F1 cells per well were seeded into 96-well plates and incubated for 24, 48, or 72 h with increasing concentrations of the peptides. No significant effect on cell proliferation was found (Fig. 2A). For adhesion assay, non-tissue culture plates were coated with matrix proteins. Fifty thousand B16F1 cells per well were seeded into 96-well plates and incubated for 30 min. NC1(XIX) peptide (30 μmol/L) was added to the culture medium during the adhesion assay. No effect of NC1(XIX) was found under these conditions (Fig. 2B). In the second set of

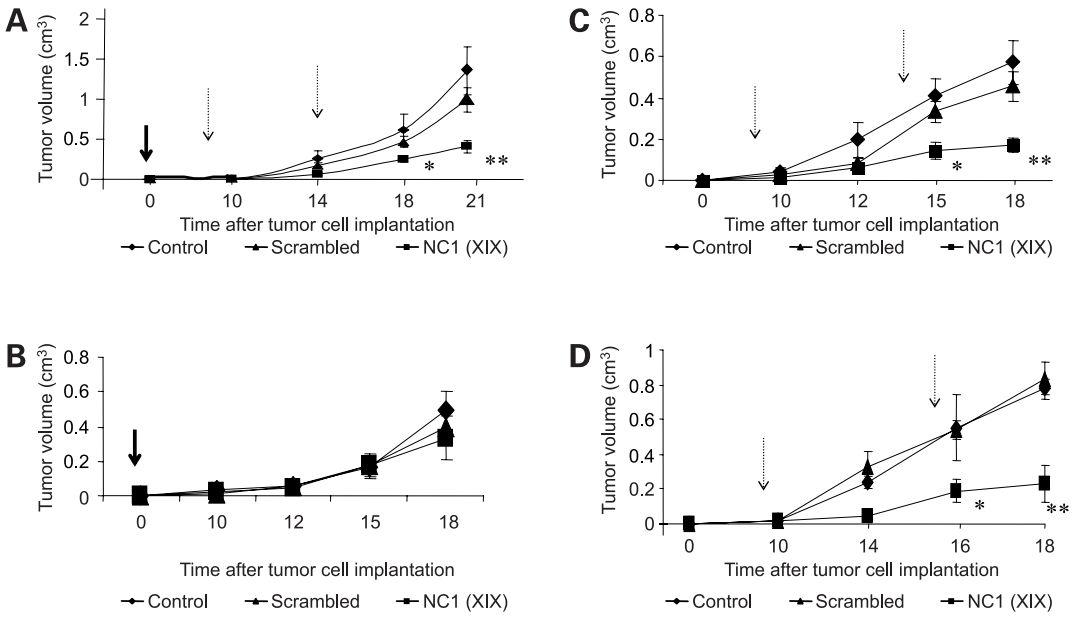

Figure 1. Inhibition of *in vivo* melanoma tumor growth by NC1(XIX) peptide. B16F1 cells (2.5×10^5 per mouse) were s.c. injected to syngeneic C57Bl6 mice. Tumor size was measured at days 7, 10, 14, 18, and 21. Kinetics of tumor growth in C57Bl6 mice treated with the NC1(XIX) peptide (■), the scrambled peptide (▲), or RPMI 1640 alone (controls; ♦). **A,** B16F1 cells were preincubated for 15 min with either control medium, NC1(XIX) peptide (30 μmol/L), or the scrambled peptide (30 μmol/L) before s.c. injection. Control medium, NC1 α1(XIX) peptide (2.5 mg/kg mouse weight), or scrambled peptide (2.5 mg/kg mouse weight) was reinjected around the tumor at days 7 and 14. **B,** B16F1 cells were only preincubated for 15 min with either control medium, NC1(XIX) peptide (30 μmol/L), or the scrambled peptide (30 μmol/L) before s.c. injection. No reinjections were done. **C,** control medium, NC1(XIX) peptide (2.5 mg/kg mouse weight), or scrambled peptide (2.5 mg/kg mouse weight) was injected around the tumor at days 7 and 14. No preincubation of tumor cells with the peptide was done. **D,** control medium, NC1(XIX) peptide (10 mg/kg mouse weight), or scrambled peptide (10 mg/kg mouse weight) was injected i.p. at days 10 and 15 after appearance of the tumor nodules. No preincubation of tumor cells was done. ↓, cell preincubation; ↓, peptide injection. *Points,* mean; *bars,* SD; *, $P < 0.01$, significantly different from control; **, $P < 0.001$ significantly different from control.

experiments, NC1(XIX) or scrambled peptides were immobilized on the plates. Fifty thousand B16F1 cells per well were seeded into 96-well plates and incubated for 30 min. Cells were found to adhere to the peptide as well as to Matrigel (Fig. 2B).

NC1(XIX) Decreases *In vitro* Cell Migration and Matrigel Invasion

For testing the capacity of NC1(XIX) to inhibit tumor cell migration and their invasive properties, 40,000 B16F1 cells were seeded onto Transwell membranes coated (invasion assay) or not (migration assay) with Matrigel.

After 24 h (migration assay) or 48 h (invasion assay) in the absence (control) or presence of NC1(XIX) in the culture medium (30 μmol/L), migrated cells on the lower face of the membrane were counted. As shown in Fig. 3, NC1(XIX) induced a significant reduction in tumor cell migration (−38%; $P < 0.01$) and Matrigel invasion (−48%; $P < 0.002$), whereas scrambled peptide at the same concentration had no significant effect.

NC1(XIX) Decreases Tumor *In vivo* Vascularization

Histologic examination of tumor sections after staining with anti-CD31 antibody showed a strong decrease in tumor vascularization in mice treated with NC1(XIX) versus control or scrambled peptide–treated mice. Whereas many large vascular structures were observed in control and scrambled peptide–treated tumors, only very small vessels were observed in NC1(XIX)-treated tumors (Fig. 4A). In addition, counting of the CD31-labeled structures showed a highly significant decrease in the vessel number in NC1(XIX)-treated tumors: 12.0 ± 1.4 (mean ± SD) per field in the control tumors; 13.0 ± 2.9 per field in the scrambled peptide–treated tumors; and 8.0 ± 1.6 per field in the NC1(XIX)-treated tumors ($P < 0.001$).

NC1(XIX) Decreases VEGF Expression in the Tumors

Because the expression of angiogenic growth factors is of paramount importance for tumor angiogenesis, we investigated the expression of VEGF in our experimental model. Immunohistochemical analysis of the tumors using a specific monoclonal anti-VEGF antibody (Fig. 4B) showed a strong (++) or an intense (+++) VEGF expression in the majority of control (13 of 21) or scrambled peptide–treated (12 of 20) tumors. On the contrary, VEGF expression was faint or absent in most of the NC1(XIX)-treated tumors (17 of 21). The differences were statistically significant when tested by the χ^2 test ($P < 0.001$).

Western blot analysis of the VEGF protein in tumor extracts showed a 65% ($P < 0.01$) decrease in the tumors treated with NC1(XIX) compared with control or scrambled peptide–treated tumors (Fig. 4C).

NC1(XIX) Decreases *In vitro* Pseudotube Formation by Endothelial Cells

To test the effects of NC1(XIX) on angiogenesis *in vitro*, HMEC-1 endothelial cells were seeded onto Matrigel and incubated for 24 h in serum-free endothelial cell growth medium supplemented or not with NC1(XIX) peptide or the scrambled peptide (30 μmol/L each).

The presence of NC1(XIX) strongly inhibited pseudotube formation compared with control or scrambled peptide–treated cultures (Fig. 5A). Quantitative evaluation of the capillary tube number by two different methods (Fig. 5B) showed a 59% and 85% decrease ($P < 0.01$) in NC1(XIX)-incubated cultures compared with the control. The scrambled peptide had no significant effect. On the other hand, NC1(XIX) had no effect on endothelial cell proliferation *in vitro* (Fig. 5C). No morphologic changes typical of cell apoptosis, such as chromatin condensation or fragmentation and nuclear shrinkage, were observed in endothelial cells incubated with NC1(XIX) (data not shown).

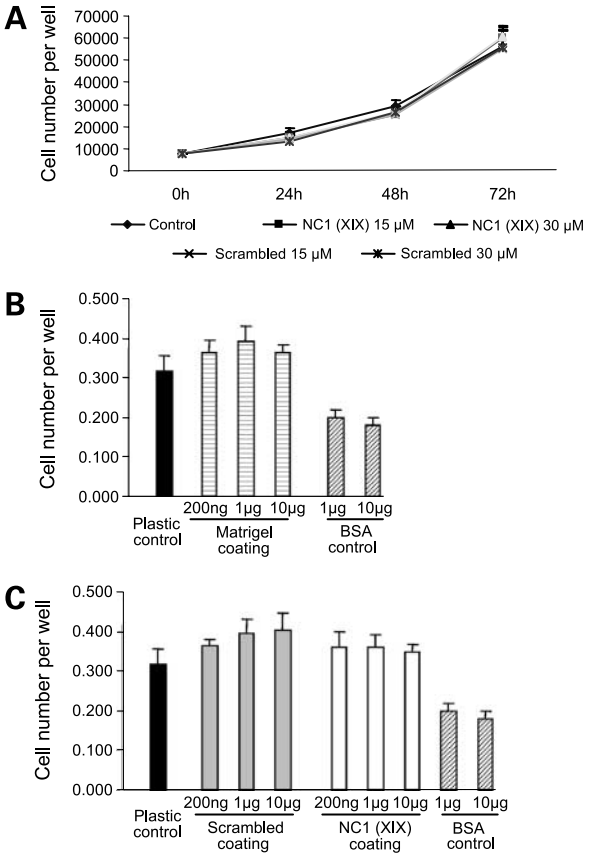

Figure 2. NC1(XIX) does not affect *in vitro* melanoma cell proliferation or adhesion. **A,** for proliferation assay, B16F1 cells were plated into 96-wells plates (10,000 per well) in RPMI 1640 supplemented with 5% fetal bovine serum and increasing concentrations of NC1(XIX) peptide. After 24, 48, or 72 h at 37°C, cell proliferation was evaluated using the WST-1 colorimetric assay. **B,** non-tissue culture plates were coated with Matrigel. Fifty thousand B16F1 cells per well were seeded and incubated for 30 min. Plates coated with BSA (1 or 10 μg per well) were used as controls in addition to plastic controls. NC1(XIX) (30 μmol/L) was added to the culture medium during the adhesion assay. The number of adhered cells was then counted. **C,** NC1(XIX) or the scrambled peptide (200 ng, 1 μg, and 10 μg per well) was immobilized on the plates and 50,000 B16F1 cells per well were seeded. Plates coated with BSA (1 or 10 μg per well) were used as control in addition to plastic controls. The number of adhered cells was counted after 30 min. *Points* and *columns,* mean; *bars,* SD.

NC1(XIX) Inhibits MT1-MMP Expression and Activity

Because MT1-MMP plays a crucial role in angiogenesis, we investigated the effects of NC1(XIX) on its expression both *in vivo* and *in vitro*.

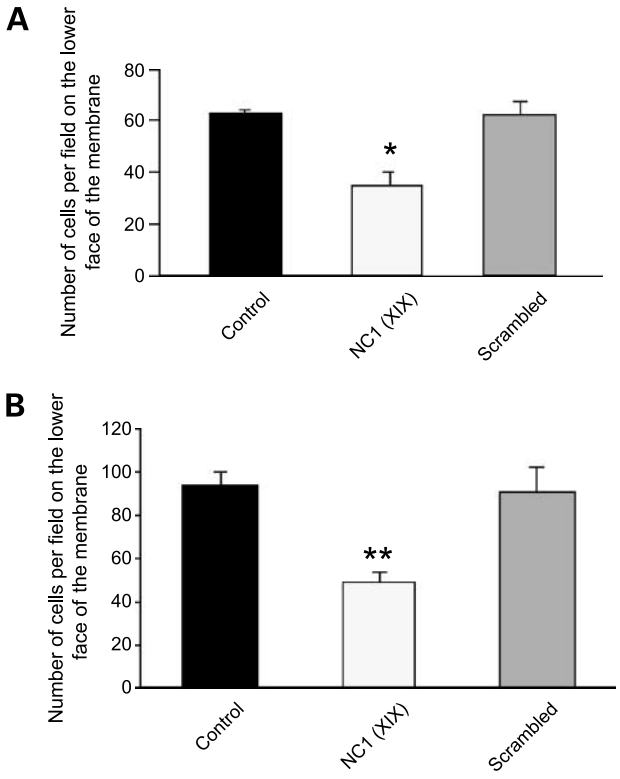

Figure 3. NC1(XIX) inhibits *in vitro* melanoma cell migration and Matrigel invasion. Migration and invasion were assayed in Transwell devices in the absence (control) or presence of NC1(XIX) or the scrambled peptide (30 μmol/L each). Forty thousand cells were suspended in serum-free RPMI and seeded onto the Transwell membranes coated (invasion assay) or not (migration assay) with Matrigel (30 $\mu g/cm^2$). At the end of the incubation period, cells were fixed with methanol and stained with crystal violet for 15 min. Cells remaining on the upper face of the membranes were scrapped and those on the lower face were counted. **A,** migration assay (24 h of incubation). **B,** Matrigel invasion assay (48 h of incubation). *Columns,* number of cells per field on the lower face of the Transwell membrane at the end of the incubation period (mean); *bars,* SD. *, $P < 0.01$, significantly different from control; **, $P < 0.001$, significantly different from control.

In vivo, immunohistochemical analysis of the tumors, using a specific anti-mouse MT1-MMP antibody (Fig. 6), showed a strong (++) or intense (+++) MT1-MMP expression in most of the control (14 of 22) or scrambled peptide–treated (10 of 18) tumors. On the contrary, MT1-MMP expression was faint or absent in most of the NC1(XIX)-treated tumors (20 of 21). The differences were statistically significant when tested by the χ^2 test ($P < 0.001$). On the other hand, NC1(XIX) did not modify MT1-MMP expression by tumor cells *in vitro* (data not shown).

For studying the effects of NC1(XIX) on MT1-MMP expression by endothelial cells *in vitro*, HMEC-1 cells were incubated for 24 h with or without NC1(XIX) or the scrambled peptide (30 μmol/L each). Real-time reverse transcription-PCR showed a strong decrease (90%; $P < 0.01$) of MT1-MMP mRNA levels in NC1(XIX)-treated endothelial cells (Fig. 7A), whereas scrambled peptide had no effect. Western blot analysis of the MT1-MMP protein showed a 36% ($P < 0.01$) decrease in endothelial cells incubated with NC1(XIX) compared with control or scrambled peptide-incubated cells (Fig. 7B). Similar data were obtained by using the Biotrak MT1-MMP activity

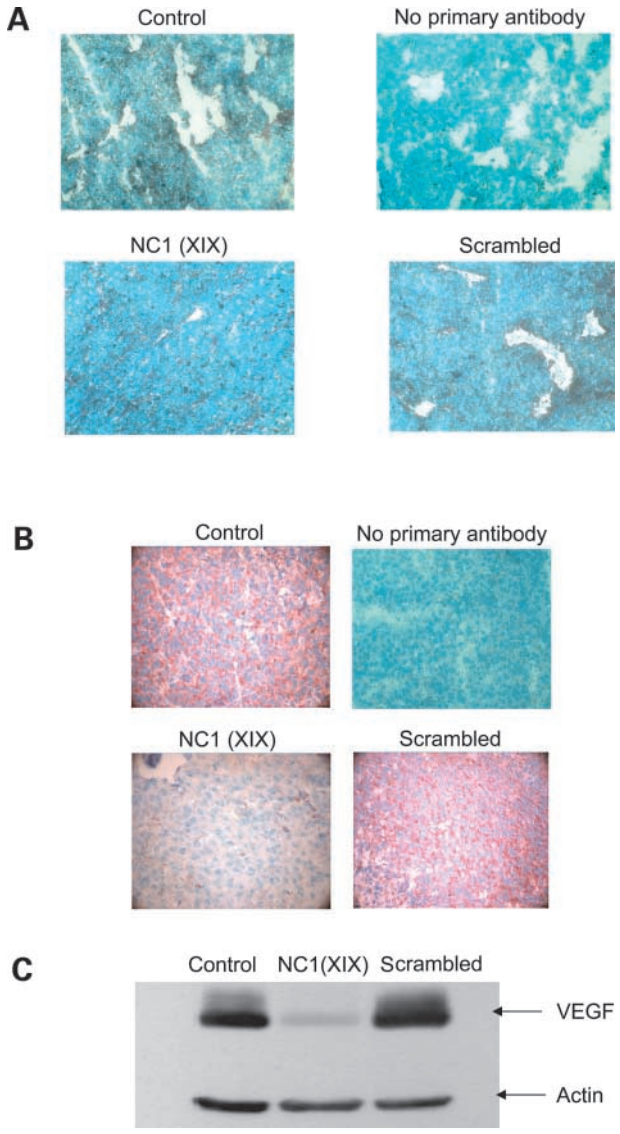

Figure 4. NC1(XIX) decreases *in vivo* tumor vascularization and VEGF expression. **A,** mice were sacrificed at day 21 and tumors were surgically extracted for morphologic studies. Tissue sections were cut at 5 μm and stained with an anti-CD31 antibody. Numerous and large vessels were seen in control and scrambled peptide–treated tumors (*arrowheads*), whereas very few large vessels were observed in NC1(XIX)-treated tumors. Representative examples of tissue sections. **B,** tumors from control, NC1(XIX)-, or scrambled peptide–treated mice were surgically extracted at day 21 for immunohistologic analysis. Tumor sections were stained with anti-VEGF antibody as described in Materials and Methods. VEGF appears as dark red staining. Tumor sections were counterstained with hematoxylin. Original magnification, ×200. **C,** Western blot analysis of VEGF expression in tumor extracts. Tumors from controls, NC1(XIX)-, or scrambled peptide–treated mice were surgically collected at day 21, and tumor extracts were prepared as described in Materials and Methods. Fifty micrograms of proteins from the extracts were deposited on the gel and analyzed by Western blot, using anti-VEGF and anti-actin antibodies.

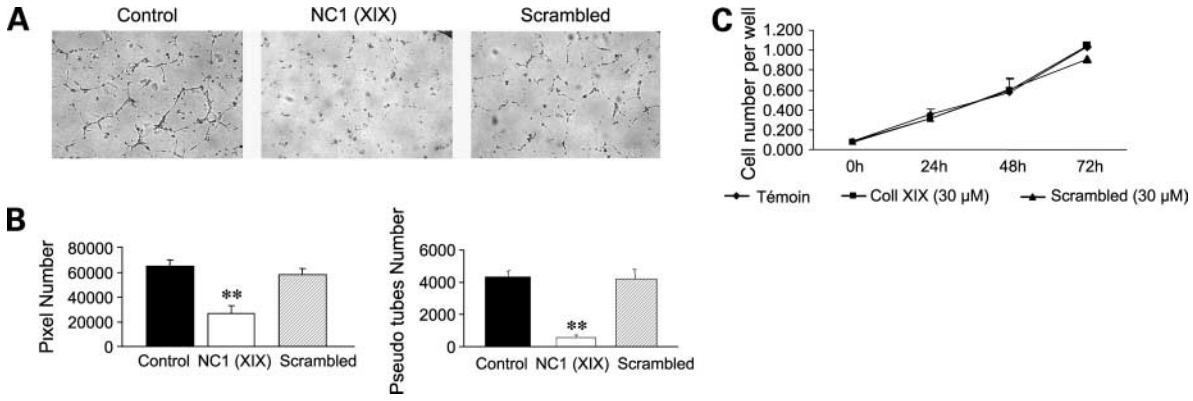

Figure 5. NC1(XIX) inhibits *in vitro* pseudotube formation but has no effect on *in vitro* endothelial cell proliferation. **A,** HMEC-1 cells (50,000 per well) were seeded on Matrigel-coated 24-well plates and incubated in the absence (control) or presence of NC1(XIX) or the scrambled peptide (30 μmol/L each). After 48 h at 37°C, pseudotube formation was photographed. Original magnification, ×10. **B,** semiquantitative evaluation of pseudotubes. Quantification was done by measuring the ratio of black pixels relative to the total pixels (*left*) and by computer analysis using the ImageJ software (*right*). Six different areas were measured for each sample. *Columns,* mean; *bars,* SD. **, $P < 0.001$, significantly different from control. **C,** HMEC-1 cells were incubated in 96 well-culture plates for 24, 48, or 72 h with either the culture medium alone (controls), NC1(XIX) (30 μmol/L), or the scrambled peptide at the same concentration. Cell proliferation was determined by the WST-1 colorimetric assay. Results were expressed as a kinetic measurement of the cell number. *Points,* mean; *bars,* SD.

assay, showing a 35% ($P < 0.01$) decrease in MT1-MMP activity in NC1(XIX)-treated HMEC-1 (Fig. 7C).

Discussion

Type XIX collagen is a minor collagen associated with the basement membrane area, together with types IV, XV, and XVIII collagens (4). It was initially characterized by its implication in muscle differentiation and morphogenesis (6, 7). This role, however, does not exclude other functions. Its basement membrane localization prompted us to hypothesize that, as it is the case for other basement membrane collagens, it might be involved in the control of tumor invasion and angiogenesis (4). In most of the already known basement membrane collagen chains, the COOH-terminal NC1 domain was shown to exhibit antitumor and/or antiangiogenic activity. This is the case for endostatin, restin, tumstatin, canstatin, and arresten, the NC1 domains of α1(XVIII), α1(XV), α3(IV), α2(IV), and α1(IV) collagen chains, respectively (8, 13). Considering these elements, we speculated that the NC1 domain of the α1(XIX) collagen

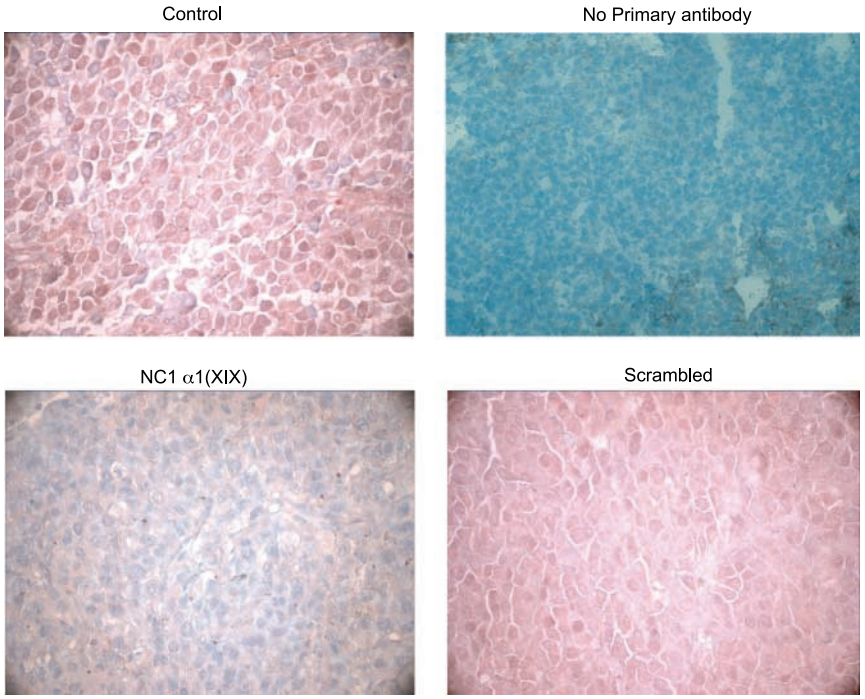

Figure 6. NC1(XIX) inhibits MT1-MMP expression in tumors. Tumors from control, NC1(XIX)-, and scrambled peptide–treated mice were extracted at day 21. Tumor sections were stained with an antimouse MT1-MMP antibody. MT1-MMP appears as dark red staining. Tumor sections were counterstained with hematoxylin. Original magnification, ×200.

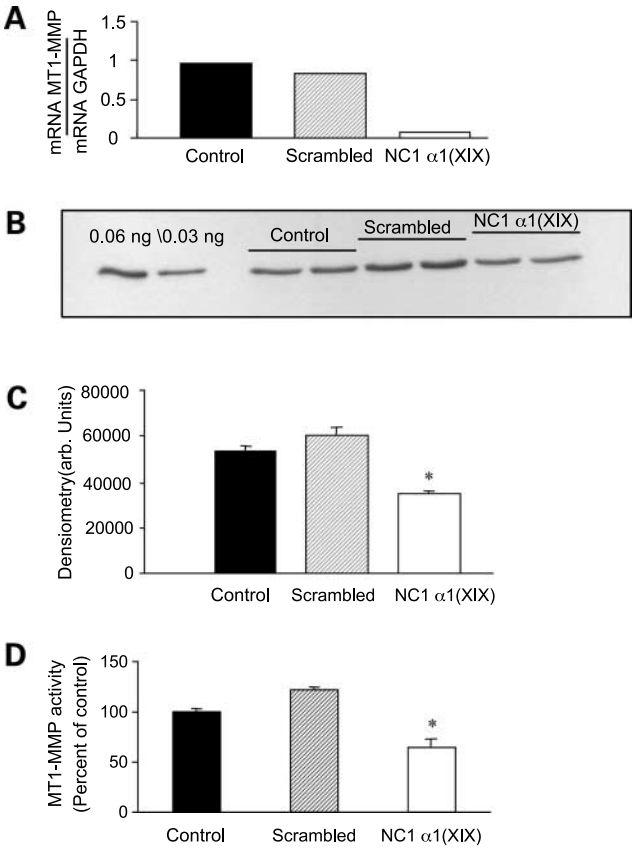

Figure 7. NC1(XIX) inhibits MT1-MMP expression by endothelial cells *in vitro*. Endothelial cells (HMEC-1) were incubated for 24 h with control medium, NC1(XIX) peptide, or the scrambled peptide(30 μmol/L) and MT1-MMP expression and activity were studied. **A,** MT1-MMP mRNA levels were measured using real-time reverse transcription-PCR analysis. Results were expressed as the target/internal standard (glyceraldehyde-3-phosphate dehydrogenase; *GAPDH*) concentration ratio of the sample divided by the target/internal standard concentration ratio of the calibrator. **B,** Western blot analysis of MT1-MMP, using a specific anti-MT1-MMP antibody. Known amounts of purified MT1-MMP (0.06 and 0.03 ng) were deposited on the first two lanes as loading controls. **C,** Western blot analysis quantification. *Columns,* mean; *bars,* SD. *, $P < 0.01$, significantly different from control; **D,** quantification of MT1-MMP activity, using the Biotrak MT1-MMP activity assay system.

chain could also be able to control tumor growth and/or angiogenesis.

To test this hypothesis, we used an experimental melanoma model obtained by s.c. injection of B16F1 melanoma cells in syngeneic C57Bl6 mice (28). In this model, injected mice develop a s.c. melanoma tumor whose volume may be easily measured. Our results show that a preincubation of melanoma cells with the NC1(XIX) peptide, followed by peritumoral reinjection of the peptide at days 7 and 14, strongly reduced tumor growth. The effect was at least as intense as that of tumstatin under the same conditions. Preincubation of melanoma cells with NC1(XIX) without peritumoral reinjections had no significant effect on tumor growth. When the initial preincubation between NC1(XIX) and tumor cells was omitted and only NC1(XIX) reinjections at days 7 and 14 were done, a strong inhibition of tumor growth was observed, nearly as intense as the full-course treatment. These results show that NC1(XIX) injections were more important than the initial preincubation for triggering tumor growth inhibition. We also observed a significant reduction in tumor growth in mice injected i.p. with 10 mg/kg NC1(XIX), without initial preincubation B16F1 cells with the peptide, showing that NC1(XIX) may also act systemically.

The antitumor activity of NC1(XIX) did not depend on an inhibition of tumor cell proliferation *in vitro*. Nevertheless, NC1(XIX) strongly inhibited *in vitro* invasive properties of B16F1 melanoma cells, as shown by migration and invasion experiments. Similar effects were observed previously for the NC1 domain of α3(IV) collagen chain (29). Histologic examination of tumor sections showed that tumor angiogenesis was significantly decreased in treated mice. This effect might depend on a direct inhibition of neoangiogenesis by NC1(XIX), as suggested by its capacity to inhibit *in vitro* pseudotube formation by human microvascular endothelial cells. Similar effects were reported previously for endostatin and canstatin (30, 31). It does not seem, however, that NC1(XIX) is able to induce endothelial cell apoptosis, as it was reported previously for other angiogenesis inhibitors because no decrease of cell number was observed when human endothelial cells were cultured in the presence of the peptide (32). On the other hand, NC1(XIX) was able to decrease VEGF expression in tumors, which may contribute to the decreased tumor vascularization that we observed.

The inhibition of MT1-MMP expression and activity by NC1(XIX) is of particular interest. MT1-MMP is up-regulated in many tumor types and is critical for pericellular degradation of the extracellular matrix, both by direct degradation of several of its components and by activating pro-MMP-2 (17, 33). In addition, MT1-MMP seems to play a central role in tumor angiogenesis and its inhibition by specific antibodies or small interfering RNA is sufficient to inhibit angiogenesis (14, 20, 21, 34, 35). Moreover, MT1-MMP was shown to be able to up-regulate VEGF expression by human breast adenocarcinoma cells and human glioma cells (36, 37). Consequently, the inhibition of MT1-MMP by NC1(XIX) might, at least partially, explain its inhibitory effects on tumor cell invasion and angiogenesis (38).

The question may be asked how NC1(XIX) may affect the expression of MT1-MMP and VEGF. Previous works have shown that other peptides from the NC1 domain of basement membrane-associated collagens may bind to specific receptor(s) of endothelial or tumor cells and activate intracellular pathways that modulate the expression of specific genes. It was the case, for instance, for endostatin, which was shown to decrease VEGF expression at the transcriptional level (39). NC1(XIX) might act similarly to endostatin. Further experiments will be, however, necessary to elucidate the intracellular signaling triggered by NC1(XIX).

Collectively, our results show that the COOH-terminal NC1 domain of human type XIX collagen exhibits antitumor properties, both *in vitro* and *in vivo*. Explaining the physiologic significance of these findings will need

further investigation. We suggest, however, that the anticancer properties of the NC1(XIX) peptide could lead to the development of new therapeutic strategies in malignant diseases.

Acknowledgments

We thank Dominique Patigny, Hervé Kaplan, and Laurence Schneider for their technical expertise.

References

1. Yoshioka H, Zang H, Ramirez F, et al. Synteny between the loci from a novel FACIT-like collagen locus (D6S228E) and α1 (XIX) collagen (COL19A1) on 6912 – 14 in human. Genomics 1992;13:884 – 6.

2. Myers JC, Sun MJ, D'Ippolito JA, Jabs EJ, Neilson EG, Dion AS. Human cDNA clones transcribed from an unusually high-molecular weight encode a new collagen chain. Gene 1993;123:211 – 7.

3. Inoguchi K, Yoshioka H, Khaleduzzaman M, Ninomiya Y. The mRNA for α 1(XIX) collagen chain, a new member of FACITs, contains a long unusual 3′ untranslated region and displays many unique splicing variants. J Biochem 1995;117:137 – 46.

4. Myers JC, Li D, Bageris A, Abraham V, Dion AS, Amenta PS. Biochemical and immunohistochemical characterization of human type XIX defines a novel class of basement membrane zone collagens. Am J Pathol 1997;151:1729 – 40.

5. Sumiyoshi H, Inoguchi K, Khaleduzzaman M, Ninomiya Y, Yoshioka H. Ubiquitous expression of the α1(XIX) collagen gene (Col19a1) during mouse embryogenesis becomes restricted to a few tissues in the adult organism. J Biol Chem 1997;272:17104 – 11.

6. Sumiyoshi H, Laub F, Yoshioka H, Ramirez F. Embryonic expression of type XIX collagen is transient and confined to muscle cells. Dev Dyn 2001; 220:155 – 62.

7. Sumiyoshi H, Mor N, Lee SY, et al. Esophageal muscle physiology and morphogenesis require assembly of a collagen XIX-rich basement membrane zone. J Cell Biol 2004;166:591 – 600.

8. Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. Cancer Res 2005;65:3967 – 79.

9. Streit M, Detmar M. Angiogenesis, lymphangiogenesis, and melanoma metastasis. Oncogene 2003;22:3172 – 9.

10. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. Cell 1991; 64:327 – 36.

11. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3:401 – 10.

12. Davis GE, Bayless KJ, Davis MJ, Meininger GA. Regulation of tissue injury responses by the exposure of matricryptic sites within extracellular matrix molecules. Am J Pathol 2000;156:1489 – 98.

13. Maquart FX, Pasco S, Ramont L, Hornebeck W, Monboisse JC. An introduction to matrikines: Extracellular matrix-derived peptides which regulate cell activity. Implication in tumor invasion. Crit Rev Oncol Hematol 2004;49:199 – 202.

14. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2:161 – 74.

15. Nagase H, Woessner JF. Matrix metalloproteinases. J Biol Chem 1999;274:21491 – 4.

16. Hofmann UB, Westphal JR, Van Kraats AA, Ruiter DJ, Van Muijen GN. Expression of integrin $\alpha_V\beta_3$ correlates with activation of membrane-type matrix metalloproteinase 1 (MT1-MMP) and matrix metalloproteinase 2 in human melanoma cells *in vitro* and *in vivo*. Int J Cancer 2000;87:12 – 9.

17. Kurschat P, Zigrino P, Nischt R, et al. Tissue inhibitor of matrix metalloproteinase-2 regulates matrix metalloproteinase-2 activation by modulation of membrane-type 1 matrix metalloproteinase activity in high and low invasive melanoma cell lines. J Biol Chem 1999;274:21056 – 62.

18. Lida J, Wilhelmson KL, Price MA, et al. Membrane type-1 matrix metalloproteinase promotes human melanoma invasion and growth. J Invest Dermatol 2004;122:167 – 76.

19. Seiki M. Membrane-type 1 matrix metalloproteinase: a key enzyme for tumor invasion. Cancer Lett 2003;194:1 – 11.

20. Bauvois B. Transmembrane proteases in cell growth and invasion: new contributors to angiogenesis? Oncogene 2004;23:317 – 29.

21. Seiki M, Yana I. Roles of pericellular proteolysis by membrane type-1 matrix metalloproteinase in cancer invasion and angiogenesis. Cancer Sci 2003;94:569 – 74.

22. Koike T, Vernon RB, Hamner MA, Sadoun E, Reed MJ. MT1-MMP, but not secreted MMPs, influences the migration of human microvascular endothelial cells in 3-dimensional collagen gels. J Cell Biochem 2002;86: 748 – 58.

23. Sounni NE, Janssen M, Foidart JM, Noel A. Membrane type-1 matrix metalloproteinase and TIMP-2 in tumor angiogenesis. Matrix Biol 2003;22: 55 – 61.

24. Bouifraden S, Drouot C, el Hadrami M, et al. Some of the amino acid chemistry going on in the laboratory of amino acids, peptides and proteins. Amino Acids 1999;16:345 – 79.

25. Wald M, Olejar T, Sebkova V, Zadinova M, Boubelik M, Pouckova P. Mixture of trypsin, chymotrypsin, and papain reduces formation of metastases and extends survival time of C57Bl6 mice with syngenic melanoma B16. Cancer Chemother Pharmacol 2001;47:16 – 22.

26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248 – 54.

27. Abramoff MD, Magelhaes PJ. Image processing with ImageJ. Biophotonics International 2004;11:36 – 42.

28. Pasco S, Ramont L, Venteo L, Pluot M, Maquart FX, Monboisse JC. *In vivo* overexpression of tumstatin domains by tumor cells inhibits their invasive properties in a mouse melanoma model. Exp Cell Res 2004;301: 251 – 65.

29. Pasco S, Han J, Gillery P, et al. A specific sequence of the noncollagenous domain of the α3(IV) chain of type IV collagen inhibits expression and activation of matrix metalloproteinases by tumor cells. Cancer Res 2000;60:467 – 73.

30. Kuo CJ, LaMontagne KR, Garcia-Cardena G, et al. Oligomerization-dependent regulation of motility and morphogenesis by the collagen XVIII NC1/endostatin domain. J Cell Biol 2001;152:1233 – 46.

31. Panka DJ, Mier JW. Canstatin inhibits Akt activation and induces Fas-dependent apoptosis in endothelial cells. J Biol Chem 2003;278: 37632 – 6.

32. Chavakis E, Dimmerler S. Regulation of endothelial cell survival and apoptosis during angiogenesis. Arterioscler Thromb Vasc Biol 2002;22: 887 – 93.

33. Hofmann UB, Westpha JR, Zendman A, Becker JC, Ruiter DJ, Van Muijen GNP. Expression and activation of matrix metalloproteinase-2 (MMP-2) and its co-localization with membrane-type 1 matrix metalloproteinase (MT1-MMP) correlate with melanoma progression. J Pathol 2000;19:1245 – 56.

34. Lafleur MA, Handsley MM, Knauper V, Murphy G, Edwards DR. Endothelial tubulogenesis within fibrin gels specifically requires the activity of membrane-type-matrix metalloproteinases (MT-MMPs). J Cell Sci 2002;115:3427 – 38.

35. Robinet A, Fahem A, Cauchard JH, et al. Elastin-derived peptides enhance angiogenesis by promoting endothelial cell migration and tubulogenesis through upregulation of MT1-MMP. J Cell Sci 2005;118: 343 – 56.

36. Sounni NE, Roghi C, Chabottaux V, et al. Up-regulation of vascular endothelial growth factor-A by active membrane-type 1 matrix metalloproteinase through activation of Src-tyrosine kinases. J Biol Chem 2004; 279:13564 – 74.

37. Sounni NE, Devy L, Hajitou A, et al. MT1-MMP expression promotes tumor growth and angiogenesis through an up-regulation of vascular endothelial growth factor expression. FASEB J 2002;16:555 – 64.

38. Deryugina EI, Soroceanu L, Strongin AY. Up-regulation of vascular endothelial growth factor by membrane-type 1 matrix metalloproteinase stimulates human glioma xenograft growth and angiogenesis. Cancer Res 2002;62:580 – 8.

39. Hajitou A, Grignet-Debrus C, Devy L, et al. The antitumoral effect of endostatin and angiostatin is associated with a down-regulation of vascular endothelial growth factor expression in tumor cells. FASEB J 2002;16:1802 – 4.