Angiogenesis, proteases and angiogenic factors during the inception of pregnancy. Crucial contributors or trivial bystanders?

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Chapter 2

Involvement of membrane-type matrix metalloproteinases in capillary tube formation by human endometrial microvascular endothelial cells. Role of MT3-MMP.

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ABSTRACT

Objective: In the endometrium angiogenesis is a physiologically process, while in most adult tissues angiogenesis is initiated only during tissue-repair or pathological conditions. Pericellular proteolysis plays an important role in angiogenesis being required for endothelial cell migration, invasion and tube formation.

Materials and Methods: We studied the expression of proteases by human endometrial microvascular endothelial cells (hEMVEC) and their involvement in the formation of capillary tubes and compared these requirements with those of foreskin MVEC (hFMVEC).

Results: Inhibition of urokinase and matrix-metalloproteinase (MMPs) both reduced tube formation in a fibrin or fibrin/collagen matrix. hEMVEC expressed various MMPs mRNAs and proteins; in particular MMP-1, MMP-2, MT1-, MT3- and MT4-MMP. MT3- and MT4-MMP mRNA expressions were significantly higher in hEMVEC than in hFMVEC. Other MT-MMP mRNAs and MMP-9 were hardly detectable. Immunohistochemistry confirmed the presence of MT3-MMP in endothelial cells of endometrial tissue. Overexpression of TIMP-1 or TIMP-3 by adenoviral transduction of hEMVEC reduced tube formation to the same extent, while only TIMP-3 was able to inhibit tube formation by hFMVEC. Tube formation by hEMVEC was partly inhibited by the presence of anti-MT-3-MMP IgG.

Conclusion: Thus, in contrast to tube formation by hFMVEC, which largely depends on MT1-MMP, capillary-like tube formation by hEMVEC is, at least in part, regulated by MT3-MMP.

INTRODUCTION

In the adult, angiogenesis plays a role in many pathological conditions, such as the growth of solid tumours, diabetic retinopathy, rheumatoid arthritis, and wound healing [Carmeliet 2003; Folkman 1995]. Physiological angiogenesis during adulthood is limited to the female reproductive tissue, namely in the ovary and endometrium. Endometrial angiogenesis plays a role in endometrial remodelling during the menstrual cycle and after conception during the implantation of the embryo [Bacharach *et al.,* 1992; Rogers, Gargett 1998; Smith 1998].

Angiogenesis is initiated by a shift in the balance between pro-angiogenic and anti-angiogenic factors [Bergers, Benjamin 2003; Hanahan, Folkman 1996]. It involves the sprouting of new capillary-like structures from existing vasculature and may involve blood-born cells that intussuscepts in and around the new vascular structures [Carmeliet 2003]. These newly formed tubes are subsequently stabilised, often by interaction with pericytes. While the general mechanisms of angiogenesis are probably rather similar in various tissues, the individual players, such as growth factors, integrins and proteases, may vary in different tissues. Endothelial cells from different tissues and vessel types have specific properties [Chi *et al.,* 2003], many of which are conserved *in vitro* [Chi *et al.,* 2003; Defilippi *et al.,* 1991; Koolwijk *et al.,* 2001]. We previously observed that different types of human microvascular endothelial cells (hMVEC) have different requirements for proliferation and capillary tube formation in vitro. While endometrial MVEC (hEMVEC) are highly sensitive to VEGF-A (VEGF) and form capillary tubules after exposure to VEGF-A, foreskin MVEC (hFMVEC) are more sensitive to bFGF and only form capillary tubes in a fibrin matrix after simultaneous exposure to bFGF or VEGF-A and the inflammatory cytokine TNFα [Koolwijk *et al.,* 1996; Koolwijk *et al.,* 2001; Kroon *et al.,* 1999].

Among the various processes that regulate angiogenesis, the generation of proteolytic activity is thought to be pivotal in the regulation of cell migration and capillary tube formation [Pepper 2001a]. Key regulators of pericellular proteolysis and capillary-like tubule formation by endothelial cells are cell-bound urokinase-type plasminogen activator (u-PA) and plasmin as well as matrix metalloproteinases (MMPs) [Bacharach *et al.,* 1992; Collen *et al.,* 2003; Galvez *et al.,* 2001; Hotary *et al.,* 2000; Koolwijjk *et al.,* 1996; Kroon *et al.,* 1999; Lafleur *et al.,* 2002; Pepper 2001a; Pepper *et al.,* 1990; Pepper 1997; Werb 1997]. Initial data on the formation of tubular structures by hEMVEC indicated that cell-bound u-PA and plasmin contribute to this process [Koolwijk *et al.,* 2001]. In addition to the u-PA/plasmin cascade, the rapidly expanding family of MMPs [Visse, Nagase 2003] plays an important role in cell migration and invasion, and in angiogenesis *in vivo* [Heymans *et al.,* 1999; Pepper *et al.,* 1990; Stetler-Stevenson 1999]. MMPs

are widely expressed in the endometrium and play a role in tissue degradation and menstrual bleeding [Salamonsen, Woodley 1996]. Furthermore, a number of them are also detected during the proliferative and early secretory phase [Tabibzadeh, Babaknia 1996], which suggests a role in endometrial remodelling and angiogenesis [Rodgers *et al.,* 1994; Smith 2001]. However, the exact role of MMPs in endometrial angiogenesis in vivo and tube formation by hEMVEC in vitro is unknown.

Membrane-type MMPs (MT-MMPs) have been suggested to play a key role in angiogenesis, in addition to the gelatinases MMP-2 and -9 [Hotary *et al.,* 2000/2002; Zhou *et al.,* 2000]. The membrane-associated localisation of membrane-type MMPs (MT-MMPs) makes this group of MMPs particularly suited to function in pericellular proteolysis [Hotary *et al.,* 2000]. Six MT-MMPs have been described: four transmembrane proteins and two GPI-anchored ones. Recently, MT1-MMP (MMP-14) received considerable attention as being involved in endothelial cell migration and invasion [Collen *et al.,* 2003; Galvez *et al.,* 2001; Lafleur *et al.,* 2002]. MT1-MMP contributes to angiogenesis by its capacity to degrade ECM components, thereby promoting cell migration, invasion and possibly the bioavailability of growth factors. Furthermore, it activates pro-MMP-2 (via the TIMP-2-MT1-MMP complex), pro-MMP-13, and $\alpha v\beta 3$-integrin, an important integrin in angiogenesis [Deryugina *et al.,* 2000; Galvez *et al.,* 2001; Sounni *et al.,* 2002; Zhou *et al.,* 2000]. MT1-MMP as well as MMP-2 are able to stimulate angiogenesis [Taraboletti *et al.,* 2002; Vagnoni *et al.,* 1998]. In hFMVEC, MT1-MMP becomes a key factor in capillary tube formation when collagen is present in the fibrinous matrix [Collen *et al.,* 2003; Hotary *et al.,* 2000]. MT2-MMP (MMP-15) and MT3-MMP (MMP-16) are also involved in cell migration and invasion, depending on the cell type [Hotary *et al.,* 2000; Shofuda *et al.,* 2001]. Their overexpression in endothelial cells can induce capillary-tube formation, similar to MT1-MMP [Hotary *et al.,* 2002]. MT1-MMP and MT2-MMP are present in endometrial tissue during various stages of the menstrual cycle; MT3-MMP mRNA is increased during the proliferative phase of the endometrium [Chung *et al.,* 2002; Goffin *et al.,* 2003; Maatta *et al.,* 2000; Zhang *et al.,* 2000]. It is generally believed that these MMPs also play a role in endometrial angiogenesis [Salamonsen 1994], but except for the expression and immunolocalisation of specific MMPs in endometrial tissue little information is available.

The activity of MMPs and MT-MMPs is regulated by activation of the pro-enzymes and by specific inhibitors, the tissue inhibitors of MMPs (TIMPs) and α-macroglobulins. The TIMP family consists of four members, which differ in expression patterns, regulation and ability to interact specifically with latent MMPs and members of the related metalloproteinases of the ADAMs and TACE group [Woessner 2001]. TIMP-1 is secreted as a soluble protein and has a general inhibiting activity on many MMPs, but does not inhibit

MT1-MMP. TIMP-3 is associated with the matrix components and has a similar inhibitory spectrum, but also inhibits MT1-MMP [Li *et al.,* 2001b]. Furthermore, TIMP-3 can induce apoptosis in various cell types [Woessner 2001].

In this study we report on the expression of MMPs and MT-MMPs by hEMVEC and the requirement of these proteases for capillary-like tube formation by these cells. By overexpressing TIMP-1 and TIMP-3 we could demonstrate that different MMPs act as key regulators for tube formation by hEMVEC and hFMVEC.

MATERIALS AND METHODS

Materials

Penicillin/streptomycin, L-glutamine and tissue culture medium 199 (M199) with 20 mM HEPES with or without phenol red were obtained from BioWhittaker (Verviers, Belgium). Newborn calf serum (NBCS) was obtained from Life Technologies (Grand Island, NY, USA). Human serum (HS), prepared from fresh blood from 10-20 healthy donors, was obtained from a local blood bank and was pooled and stored at 4°C. NBCS and HS were heat-inactivated before use. Pyrogen-free human serum albumin (HSA) was obtained from Sanquin (Amsterdam, The Netherlands). Tissue culture plastics and microtiter plates were obtained from Costar/Corning (Cambridge, MA, USA) and Falcon® (Becton Dickinson (BD) Biosciences), Bedford, MA, USA). A crude preparation of endothelial cell growth factor (ECGF) was prepared from bovine brain as described previously [Maciag *et al.,* 1979]. Heparin and thrombin were obtained from Leo Pharmaceutics Products (Weesp, the Netherlands). Human fibrinogen was obtained from Chromogenics AB (Mölndal, Sweden). Dr. H. Metzner and Dr. G. Seeman (Aventis Behring GmbH, Marburg, Germany) generously provided factor XIII. Fibronectin was a gift from Dr. J. van Mourik (CLB, Amsterdam, the Netherlands). Rat tail collagen type-I was obtained from BD Biosciences. Human recombinant vascular endothelial growth factor-A (VEGF-A) was obtained from RELIATech (Braunschweig, Germany) and tumour necrosis factor alpha (TNFα) was a gift from Dr. J. Travernier (Biogent, Gent, Belgium). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Adenoviral vectors containing LacZ, TIMP-1 and TIMP-3 were previously described [Lamfers *et al.,* 2002; Quax *et al.,* 2001; Van der Laan *et al.,* 2003]. Aprotinin was purchased from Pentapharm Ltd (Basel, Switzerland). BB94 (Batimastat) was a kind gift from Dr. E.A. Bone (British Biotech, Oxford, UK). Rabbit-anti-human polyclonal antibodies against u-PA, MMP-9 and MT1-MMP were produced in our laboratory. Mouse-anti-human monoclonal antibody against MT3-MMP was obtained from Oncogene Research Products (Boston, USA). Human recombinant MT1-MMP (pro-domain-catalytic domain-hemopexin

domain) was purchased from Chemicon (Temecula, CA, USA) and recombinant pro-MMP-9 from Invitek (Berlin, Germany). PBS/T concentrate was obtained from Organon Teknika (Boxtel, Holland). GAPDH control reagents (VIC-labeled) were purchased from Applied Biosystems (Nieuwerkerk a/d/ IJssel, the Netherlands). For western blotting, protease inhibitors from Roche Diagnostics (Almere, the Netherlands), Immobilon-P polyvinylidene fluoride transfer membranes from Milipore (Bedford, USA), skim milk powder from Merck (Amsterdam, the Netherlands), goat anti-β-actin antibody (sc-1615) and horseradish peroxidase-conjugated secondary antibodies from Santa Cruz (Heerhugowaard, The Netherlands) were used. The Super Signal West Dura Extended Duration Substrate (Pierce, St. Augustin, Germany) and the luminescent image workstation (Roche Diagnostics, Almere, the Netherlands) were used for visualisation.

Cells

Human endometrial microvascular endothelial cells (hEMVEC) were isolated from endometrial tissue from pre-menopausal women and cultured and characterised as previously described [Koolwijk *et al.,* 2001]. hEMVEC were maintained in hEMVEC culture medium: M199 without phenol-red supplemented with 20 mM HEPES (pH 7.3), 20% HS, 10% NBCS, 150 μg/mL ECGF, 5 U/mL heparin, 100 IU/mL penicillin and 100 mg/mL streptomycin. The cells were cultured on fibronectin-coated dishes under humidified 5% CO_2 / 95% air atmosphere. VEGF-A (5 ng/mL) was added to the culture medium of the primary isolates to facilitate the initial growth of the endothelial cells. Human foreskin microvascular endothelial cells (hFMVEC) were isolated, characterised and cultured as previously described [Defilippi *et al.,* 1991; Van Hinsbergh *et al.,* 1987].

In vitro capillary-like tube formation assay

Human fibrin matrices were prepared as described before [Koolwijk *et al.,* 2001]. For the collagen gels, 7 volumes of rat tail collagen type-I (3 mg/mL) were mixed with 1 volume of 10x M199 with phenol red and 2 volumes of 2% (w/v) Na_2CO_3 (final pH 7.4). 300μl Aliquots were added to each well of a 48-wells plate and allowed to gelate at 37 °C in the absence of CO_2.

Confluent hEMVEC were detached and seeded at a split ratio of 2:1 on top of the fibrin and/or collagen matrices and cultured for 24 h hEMVEC culture medium without ECGF and heparin. Subsequently, the endothelial cells were cultured with the mediators indicated for 2 - 5 days. Invading cells and the formation of capillary-like structures of endothelial cells in the three-dimensional fibrin and/or collagen matrix were analysed by phase contrast microscopy. The total length of the structures formed was measured in 6 randomly chosen microscopic fields (7.3 mm^2/field) by computer-equipped Optimas image analysis software (Bioscan, Demons, WA) connected to a monochrome CCD camera (MX5) and expressed as mm/cm^2.

Gelatin zymography

Gelatinolytic activities of MMPs secreted by hEMVEC were analysed by zymography on gelatin-containing polyacrylamide gels as described [Birkedal-Hansen, Taylor 1982]. Using this technique both active and latent species can be visualised. Samples were applied to a 10% (w/v) acrylamide gel co-polymerised with 0.2% (w/v) gelatin. After electrophoresis the gels were washed three times for 10 min in 50 mmol/L Tris/HCl, pH 8.0, containing 5 mmol/L $CaCl_2$, 1µmol/L $ZnCl_2$ and 2.5% (w/v) Triton X-100 to remove the SDS, followed by three washes of 5 min in 50 mmol/L Tris/HCl, pH 8.0, containing 5 mmol/L $CaCl_2$, 1µmol/L $ZnCl_2$ and incubated overnight at 37fflC. The gels were stained with Coomassie Brilliant Blue R-250.

Immunohistochemistry

Immunohistochemical staining of MT3-MMP was performed in paraffin-embedded sections of human endometrium using a monoclonal mouse anti-MT3-MMP and a horseradish peroxidase-conjugated horse-anti-mouse antibody. Specificity of the immunohistochemical reaction was verified by omission of the first antibody as wells as using normal mouse serum in stead of the first antibody.

Western blotting

Total cellular extracts were prepared in the presence of protease inhibitors and applied to SDS-PAGE electrophoresis essentially as described [Kleemann *et al.,* 2003]. After proteins were blotted onto Immobilon-P polyvinylidene fluoride transfer membranes, the blots were blocked with 5% (w/v) skim milk powder diluted in 20 mM Tris (pH 7.4), 55 mM NaCl, and 0.1% (v/v) Tween-20. Then, blots were incubated with a mouse anti-MT3-MMP antibody or a goat anti-β-actin antibody followed by horseradish peroxidase-conjugated secondary antibodies. All antibodies were diluted in 20 mM Tris (pH 7.4), 55 mM NaCl, 0.1% (v/v) Tween-20, and 5% (w/w) bovine serum. The Super Signal West Dura Extended Duration Substrate and the luminescent image workstation were used for visualization.

RNA Isolation and real-time RT-PCR

Total RNA from hEMVEC and hFMVEC was isolated as described previously [Chomczynski, Sacchi 1987]. Reverse transcription (RT) was carried out in 20 µl volumes using random primers and a cDNA synthesis kit purchased from Promega. MMP and MT-MMP expression was quantified using real-time PCR according to the Taqman method of Applied Biosystems (Perkin Elmer) using a forward and reverse primer combined with a specific (6-carboxy-fluorescein/6-carboxy-tetramethyl-rhodamine [FAM/TAMRA]) double-labelled probe. The following sequences were used for MT3-MMP (MMP-16): forward primer, 5'-GGC TCG TGT GGG AAA TGG TA-3'; reverse primer, 5'-AGA ACT CTT

CCC CCT CAA GTG-3'; and probe, 5'-ACA GCT GGC TCT ACT TCC CCA TGG C-3'. Primers and probes for MT1-MMP were described previously [Collen *et al.,* 2003]. All data were controlled for quantity of RNA input by performing measurements on the endogenous reference gene GAPDH (VIC-labelled) as follows. For each RNA sample, a difference in Ct values (dCT) was calculated for each mRNA by taking the mean Ct of duplicate wells and subtracting the mean Ct of the duplicate wells for the reference RNA GAPDH measured in the same RT reaction. All RT reactions were carried out in quadruplicate. As positive controls were used: cDNA of human endometrial stromal cells for MMP-12, cDNA of HT1080 cells for MMP-13 and ds cDNA encoding for MMP-3, MMP-7 and MMP-8.

Adenoviral gene transfer of TIMP-1 and TIMP-3 to hEMVEC and hFMVEC

Replication-deficient adenoviral vectors (E1-deleted, transcriptional control via the CMV promotor) encoding human TIMP-1 (AdTIMP-1), human TIMP-3 (AdTIMP-3) and a β-galactosidase-encoding adenoviral vector (AdLacZ), as a control, were used for the experiments [Quax *et al.,* 2001]. Confluent hEMVEC and hFMVEC were washed twice with M199 supplemented with 0.1% HSA to remove human serum components, subsequently the hEMVEC were incubated with the adenoviral constructs in M199 containing 0.1% HSA for 2 hours. After transduction the medium was replaced with hEMVEC culture medium without VEGF-A. 24 h later the cells were seeded on top of a three-dimensional fibrin/fibrin-collagen matrix and stimulation was started 6 h after seeding.

TIMP-1 ELISA and MMP Bioactivity Assays

TIMP-1 antigen was assayed by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Oxon, United Kingdom). MT1-MMP and MMP-9 activity were determined by MMP activity assays (Biotrak; Amersham, Biosciences, Buckingshamshire, UK) as previously indicated [Collen *et al.,* 2003; Hanemaaijer *et al.,* 1998]. Selective TIMP-3 activity over that of TIMP-1 was assayed by determination of active MT1-MMP in extracts of hEMVEC transduced with AdLacZ, AdTIMP-1, and AdTIMP-3. Inhibition of MMP-9 activity by TIMP-1 and TIMP-3 was determined by addition of serial dilutions of 48-hour conditioned media of hEMVEC transduced with AdLacZ, AdTIMP-1, and AdTIMP-3 to APMA activated recombinant pro-MMP-9.

Statistics

Experiments were performed with duplicate wells and expressed as mean ± SEM. For statistical evaluation the analysis of variance (ANOVA) was used, followed by a modified t-test according to Bonferroni. Statistical significance was accepted at $p<0.05$.

RESULTS

Capillary-like tube formation by hEMVEC is inhibited by collagen type-I

Three-dimensional matrices were prepared consisting of pure fibrin, collagen or mixtures of fibrin and collagen. As previously reported [Koolwijk *et al.,* 2001], hEMVEC form spontaneously capillary-like tubular structures in a fibrin matrix, a process that is markedly enhanced by VEGF-A (Figure 1A, C). When hEMVEC were seeded on top of matrices

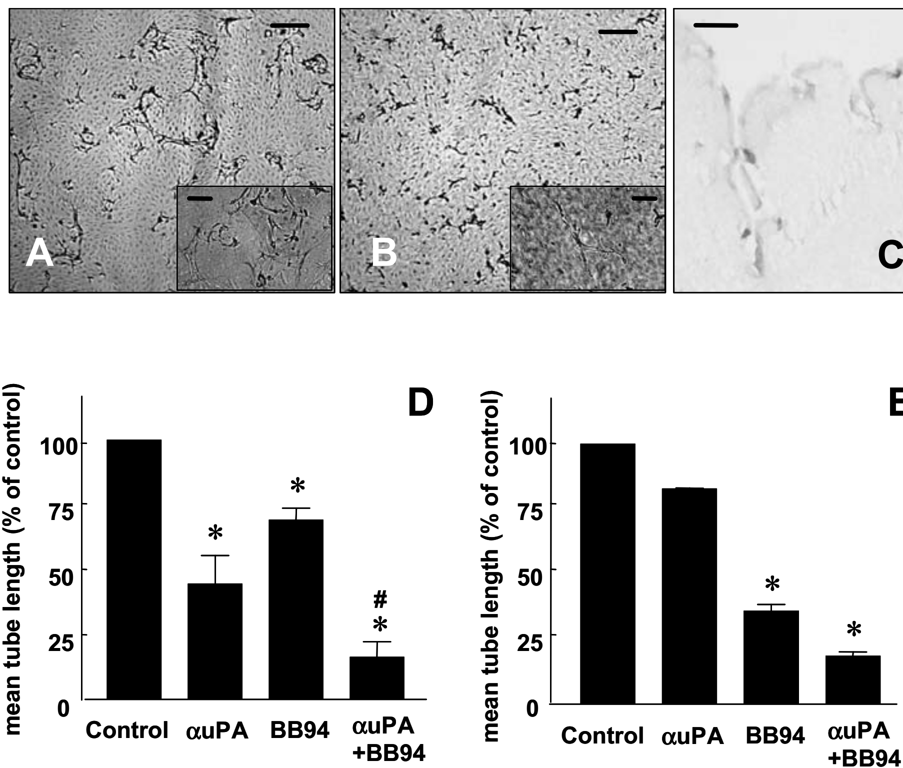

Figure 1. Capillary-like tube formation by hEMVEC in a fibrin or collagen matrix depends on u-PA and MMP activities.
hEMVEC were cultured on top of a three-dimensional fibrin matrix (A,C,D) or 50-50% fibrin/collagen-type-1 matrix (B,E) and stimulated with VEGF-A (10 ng/mL). A and B: Micrographs taken after 4 days of culturing; insets in A and B show details of capillary-like structures. Bar = 300 μm, Bar insets = 100 μm. C: Cross section perpendicular to the matrix surface and stained with Hematoxylin-Phloxine-Safran (bar = 50 μm). D and E: hEMVEC were cultured with 10 ng/mL VEGF-A (control) in the absence or presence of polyclonal anti-u-PA (αuPA, 100 μg/mL), BB94 (5 μg/mL) or a combination of BB94 and anti-u-PA. After 3-5 days of culturing, mean tube length was measured by image analysis. The data in panel D are expressed as a percentage of VEGF-A-induced tube formation ± SEM of 6 independent experiments of duplicate wells performed with 3 different hEMVEC isolations. Panel E represents 3 experiments. *: *$P<0.05$ vs. control, #: $P<0.05$ vs. αuPA.*
(see colour figures supplement)

containing 0-50% type-I collagen homogeneously mixed with fibrin, a concentration-dependent decrease in the extent of tube formation was seen. In a mixed collagen-fibrin matrix (50/50), the decrease was 55±4% under basal conditions (n = 3, not shown) and 53±2% in the presence of VEGF-A (Figure 1B) as compared to the tube formation in a pure fibrin matrix (Figure 1A). In a pure collagen type-I matrix, capillary-like structure formation by hEMVEC was hardly detectable, even after stimulation with VEGF-A (data not shown).

U-PA/plasmin and MMPs are involved in tube formation by hEMVEC in matrices composed of fibrin and/or collagen

To establish the involvement of u-PA/plasmin and MMPs in the formation of capillary-like structures by hEMVEC, u-PA-blocking antibodies, the plasmin inhibitor aprotinin, or the broadly acting metalloproteinase inhibitor BB94 were used (Figure 1C and D). The VEGF-A-enhanced tube formation in a fibrin matrix was reduced by 55±11% by u-PA-blocking antibodies (Figure 1D), and by 54±7% by the plasmin inhibitor aprotinin (data not shown). In a matrix consisting of an equal mixture of fibrin and collagen anti-u-PA antibodies reduced tube formation only by 17±0% (Figure 1E). The inhibiting effect of BB94 was increased by adding collagen, since tube formation in pure fibrin was inhibited by 31±5% and in collagen-fibrin matrices by 64±3%. An almost complete inhibition (84±6% and 82±2%, respectively) of capillary-like structure formation was seen after the simultaneous addition of BB94 and anti-u-PA antibodies (Figure 1D and E).

Table 1. mRNA level of MT-MMPs in hEMVEC and hFMVEC.

		hEMVEC		hFMVEC	
		CT	dCT	CT	dCT
Transmembrane MT-MMPs	**MT1-MMP**	27.8 ± 0.4	9.0 ± 0.4	27.3± 0.9	8.4 ± 0.4
	MT2-MMP	33.7 ± 1.2	14.1 ± 1.2	35.9 ± 1.6	16.6 ± 1.3
	MT3-MMP	26.4 ± 0.2	7.4 ± 0.4*	27.8 ± 0.8	9.1 ± 0.4
	MT6-MMP	36.3 ± 1.0	17.7 ± 0.8	34.5 ± 1.1	15.6 ± 1.8
GPI-anchored MT-MMPs	**MT4-MMP**	26.9 ± 0.2	7.3 ± 0.3 *	30.2 ± 1.2	10.6 ± 0.6
	MT5-MMP	31.6 ± 0.3	12.8 ± 0.3	33.8 ± 2.1	14.6 ± 1.4

Table 1. Analysis of MT-MMP mRNA expression in VEGF-A-stimulated hEMVEC and hFMVEC.
Confluent hEMVEC and hFMVEC were stimulated with 10 ng/ml VEGF-A for 24 hours. After stimulation, RNA was isolated and cDNA was synthesized as described. Real-time RT-PCR for MT-MMP/GAPDH pairs were performed as described and expressed as the number of cycles (CT ± SEM). The housekeeping gene GAPDH was used to correct for the total mRNA content of the samples. The dCT values were calculated as the difference in number of cycles required for the PCR reaction to enter logarithmic phase and expressed as dCT ± SEM. The gene expression of MT3-MMP and MT4-MMP mRNA was significantly higher in hEMVEC compared to the expression in hFMVEC (*: P<0.01). The gene expression of the other MT-MMPs was comparable between the two cell types.

hEMVEC express various MMPs and MT-MMPs

To study which MMPs are expressed by hEMVEC, real-time RT-PCR was used to assess the expression and regulation of MMP mRNA levels in hEMVEC. Real-time RT-PCR revealed that hEMVEC express considerable amounts of MMP-1, MMP-2, MT1-MMP, MT3-MMP and MT4-MMP mRNAs (i.e. less than 30 cycles and dCT< 9) under basal as well as VEGF-A-stimulated conditions. The data for the MT-MMPs are given in Table 1. hFMVEC had a similar expression pattern as hEMVEC, except for MMP-1, which was poorly expressed by hFMVEC under basal conditions (not shown), and MT3-MMP and MT4-MMP, which were expressed to a higher degree in hEMVEC (Table 1). Under basal and VEGF-A-stimulated conditions hEMVEC expressed relatively small amounts of MMP-9 (mean CT = 35.3 ± 1.5 cycles; mean dCT 14.4 ± 1.3 (± SEM)) and MT2-, MT5- and MT6-MMP (Table 1). The MMP-9 mRNA expression increased markedly when the cells were stimulated with 10^{-8} M phorbol ester PMA (mean CT 27.4 ± 1.0; dCT 8.4 ± 0.7 (± SEM)). No mRNA of MMP-3, MMP-7, MMP-8, MMP-12 and MMP-13 was detected in

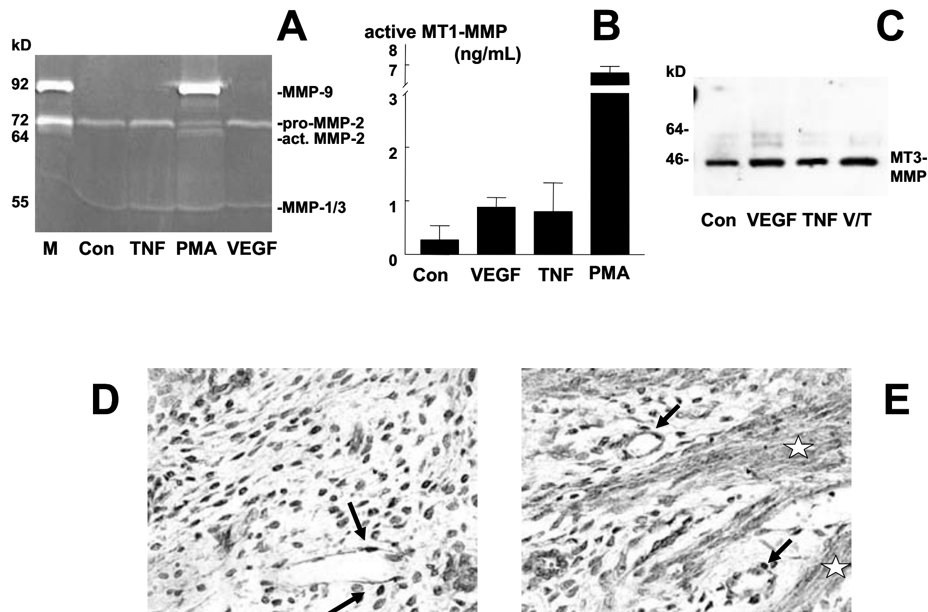

Figure 2. hEMVEC express various MMPs and MT-MMPs.
hEMVEC were cultured for 24 h in M199 supplemented with 0.5% HSA (A) or 20% HS (B,C) and were not stimulated (control) or stimulated with TNFα (2.5 ng/mL), VEGF-A (10 ng/mL) or PMA (10^{-8} M), as indicated. A: Gelatin zymography of 24 h conditioned medium. (M = ladder) B: MT1-MMP activity in cell lysates (mean ± range of two experiments performed in duplicate wells with two different isolations; detection limit of the assay 0.2 ng/mL). C: Western blot of MT3-MMP in 24 h conditioned medium. D and E: Immunohistochemical analysis of MT3-MMP in endometrial tissue shows the presence of MT3-MMP in endothelial cells (D, arrows) and myometrium (E, stars).
(see colour figures supplement)

hEMVEC. Positive controls resulted in abundant signals: ds cDNA encoding for MMP-3, MMP-7 and MMP-8, cDNA of human endometrial stromal cells for MMP-12 and cDNA of HT1080 cells for MMP-13.

The expression of active MMPs was confirmed by gelatin zymography and activity assays. Gelatin zymography of serum-free hEMVEC-conditioned media (24 h) showed expression of latent MMP-2 (72 kDa) and a 55kDa band that represents MMP-1 or MMP-3. From the mRNA data we assume that the 55kDa band represents MMP-1 rather than MMP-3. Stimulation with 10^{-8} M PMA induced MMP-9 (92kDa) protein synthesis and activation of MMP-2 (64 kDa, Figure 2A). The presence of MT1-MMP was demonstrated by activity assay (Figure 2B). Both VEGF-A and TNFα exposure doubled the activity of MT1-MMP, while phorbol ester caused a dramatic increase in MT1-MMP activity in hEMVEC (Figure 2B). The presence of MT3-MMP protein was confirmed by Western blotting. The production of MT3-MMP was not affected by TNFα and increased slightly after VEGF-A exposure (Figure 2C). MT3-MMP was detectable in endothelial cells of proliferative human endometrial tissue, as well as in endometrial epithelial cells and myometrial cells (Figure 2D and E).

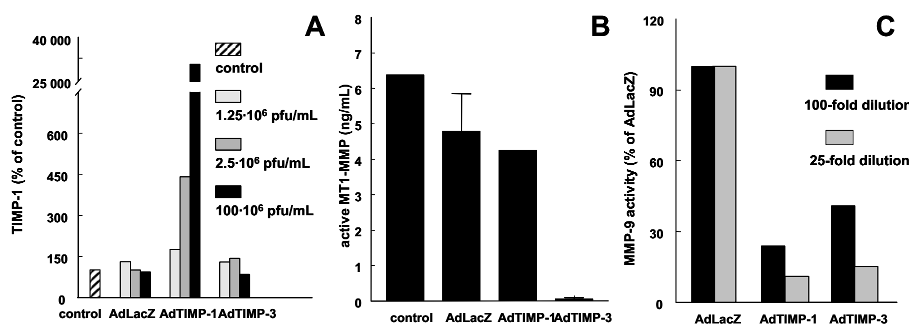

Figure 3. hEMVEC overexpress active TIMP-1 and -3 after transduction.
Confluent hEMVEC were transduced with $1.25 \cdot 10^6$, $2.5 \cdot 10^6$ and $1.0 \cdot 10^8$ pfu/mL AdLacZ, AdTIMP-1 or AdTIMP-3 as described in the Methods section. After 2 h the medium was removed and the cells were incubated for 6 h with hEMVEC culture medium and incubated for 48 h in M199 supplemented with 0.5 % HSA, 10 ng/mL VEGF-A with or without PMA (10^{-8} M). A: TIMP-1 levels were determined in the hEMVEC-conditioned medium by ELISA following the manufacturers' descriptions. B: MT1-MMP activity in the lysates of PMA-stimulated transduced hEMVEC was inhibited by TIMP-3 and not by TIMP-1. MT1-MMP activity was analysed as indicated in the Methods section. C: MMP-9 activity was assayed using a bioactivity assay as described in the Material and Methods. The ability of TIMP-1 and TIMP-3 to inhibit MMP-9 activity was measured in 100- (black bars) and 25-fold (gray bars) dilutions of the conditioned media of hEMVEC overexpressing TIMP-1 orTIMP-3.

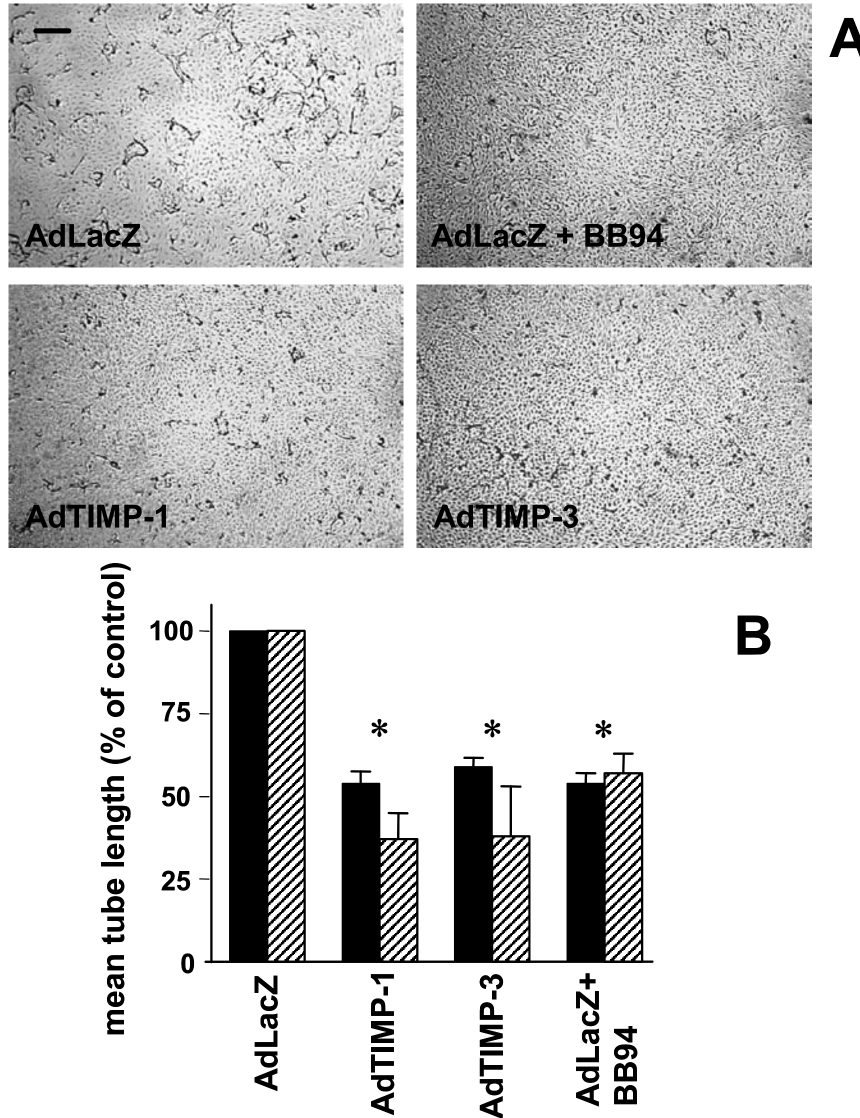

Figure 4. Both TIMP-1 and TIMP-3 inhibit capillary-like tube formation by hEMVEC.
hEMVEC were transduced with $2.5 \cdot 10^6$ pfu/mL AdLacZ, AdTIMP-1 and AdTIMP-3 and were cultured on top of a three-dimensional fibrin matrix or a fibrin-10% collagen matrix and stimulated with VEGF-A (10 ng/mL) with or without BB94 (5 μg/mL). A: Phase contrast micrographs after 3 days of culturing showing tube formation in the fibrin matrix, Bar = 300 μm. B: Mean tube length was measured and expressed as a percentage of the tube formation by the AdLacZ-transduced cells ± SEM/range of 5 (fibrin matrix, black bars) and 2 (fibrin-collagen matrix, striped bars) independent experiments performed in duplicate wells. The mean tube length of the AdLacZ-transduced hEMVEC was 239±13 mm/cm² on the fibrin-collagen matrix. * *P<0.03 vs LacZ transduced cells.*

Adenoviral gene transfer of both TIMP-1 and TIMP-3 impairs VEGF-A-induced tube formation by hEMVEC

As the general metalloproteinase inhibitor BB94 inhibited tube formation by hEMVEC, the effects of TIMP-1 and TIMP-3, two physiological tissue inhibitors of MMPs, on this process were studied. hEMVEC were infected for 2h with replication-deficient adenoviruses expressing human TIMP-1 (AdTIMP-1), TIMP-3 (AdTIMP-3) or a control LacZ (AdLacZ). Transduction of hEMVEC with AdTIMP-1 caused a concentration-dependent increase in TIMP-1 antigen production, while AdLacZ or AdTIMP-3 did not affect TIMP-1 production (Figure 3A). To verify whether the overexpressed TIMP-1 and -3 were func-

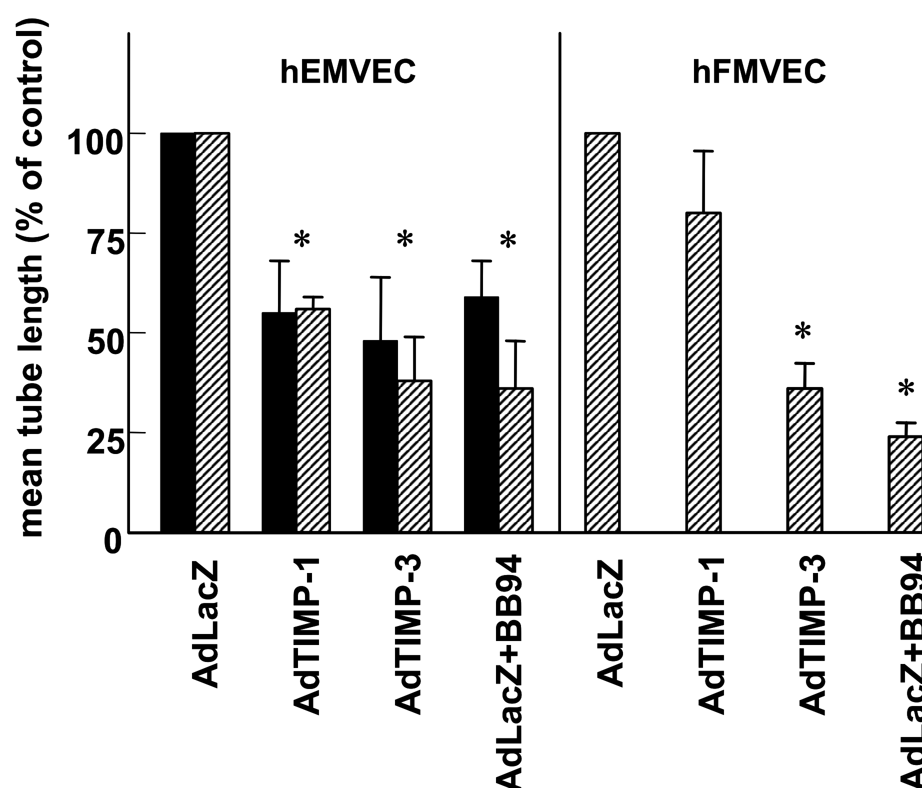

Figure 5. TIMP-1 inhibits capillary-like tube formation by hEMVEC but not by hFMVEC.
hEMVEC and hFMVEC were transduced with $2.5 \cdot 10^6$ pfu/mL AdLacZ, AdTIMP-1 and AdTIMP-3. Subsequently the cells were cultured on top of a three-dimensional fibrin matrix or a fibrin-10% collagen matrix in M199 supplemented with 10% HS and 10% NBCS and stimulated with VEGF-A (10 ng/mL) and TNFα (10 ng/mL) with or without BB94 (5 μg/mL). Mean tube length was measured and expressed as a percentage of the tube formation by the AdLacZ-transduced cells ± SEM/range of 2-3 independent experiments performed in duplicate or triplicate wells (fibrin matrix; black bars, fibrin-collagen matrix; striped bars). The mean tube length of the AdLacZ-transduced hEMVEC was 270±80 mm/cm^2 on the fibrin matrix and 266±83 mm/cm^2 on the fibrin-collagen matrix, * *P<0.05 vs. LacZ transduced cells.*

tional and active, their effect on MT1-MMP and MMP-9 activity was analysed. In contrast to cell extracts of AdLacZ- or AdTIMP-1-transduced-hEMVEC, in which MT1-MMP remained active, MT1-MMP activity was completely inhibited in cell extracts of AdTIMP-3-transduced-hEMVEC (Figure 3B). AdTIMP-1- and AdTIMP-3-transduced hEMVEC inhibited exogenous active MMP-9 comparably (Figure 3C).
Previous studies on HUVEC and hFMVEC have shown that TIMP-3 was a more potent inhibitor of capillary-tube formation than TIMP-1 [Lafleur *et al.,* 2002; Collen *et al.,* 2003; Anand-Apte *et al.,* 1997]. Unexpectedly, in hEMVEC both TIMP-1 and TIMP-3 overexpression inhibited VEGF-A-induced tube formation, to an extent similar as BB94 (Figure 4A and B). This was found both in fibrin and in fibrin-collagen matrices (Figure 4B). No apparent cell death or morphological changes were observed either in the AdTIMP-1- or AdTIMP-3-transduced hEMVEC.

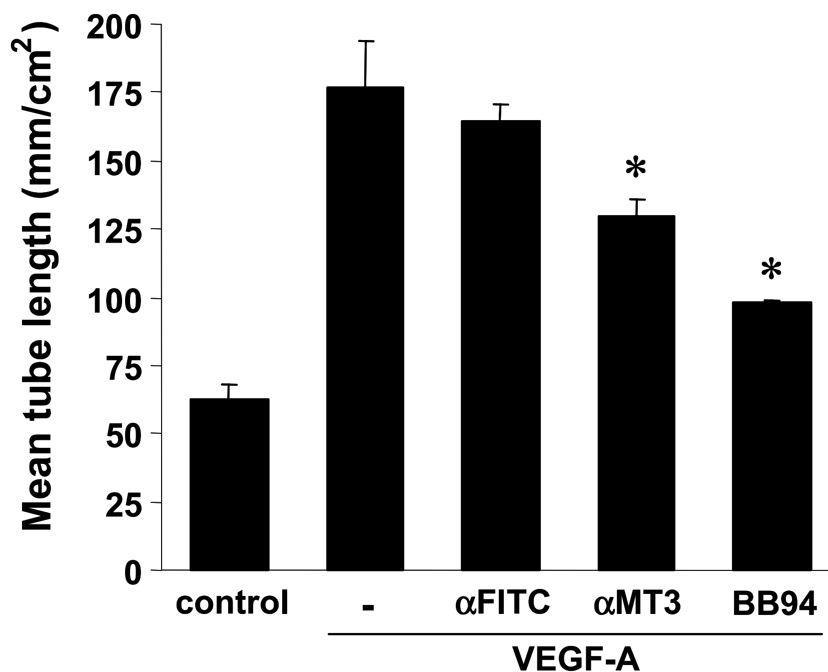

Figure 6. Inhibition of MT3-MMP reduces tube formation by hEMVEC.
hEMVEC were cultured on top of a three-dimensional fibrin matrix in M199 supplemented with 10% HS and 10% NBCS. Cells were cultured under control conditions in the presence of 0.5 ng/ml VEGF or stimulated with VEGF-A (10 ng/mL), VEGF-A (10 ng/mL) and anti-FITC IgG (25 μg/ml), VEGF-A (10 ng/mL) and anti MT3-MMP IgG (αMT3; 25 μg/ml) or VEGF-A (10 ng/mL) and BB94 (5 μg/mL). Mean tube length was measured and expressed as the mean tube length ± SEM of 2 experiments performed in duplicate wells. * *P<0.02 vs VEGF stimulated hEMVEC; P<0.05 vs VEGF /anti-FITC IgG treated hEMVEC.*

Comparison of the effect of TIMP-1 and TIMP-3 overexpression on tube formation by hEMVEC and hFMVEC

Because of the lack of effect of TIMP-1 on tube formation in our previous experiments with VEGF/TNFα-stimulated hFMVEC [Collen *et al.,* 2003], we compared the effects of TIMP-1 and TIMP-3 overexpression on capillary-like tube formation by hEMVEC and hF-MVEC under identical culture conditions. Both cells types were grown on a fibrin-10% collagen matrix and stimulated by the simultaneous addition of VEGF and TNFα, which is required to induce tubules by hFMVEC [Koolwijk *et al.,* 1996]. Like in VEGF-stimulated hEMVEC, both TIMP-1 and TIMP-3 reduced capillary-like tube formation in VEGF/TNFα-stimulated hEMVEC to the same extent as BB94 (Figure 5, stripped bars). In contrast, only TIMP-3 inhibited tube formation by hFMVEC to a significant extent. Similar data were obtained with fibrin matrices (Figure 5, black bars). No significant cell detachment was observed in the AdTIMP-1- or AdTIMP-3-transduced hFMVEC and hEMVEC grown on the fibrin matrix, neither under control conditions nor in cells stimulated with VEGF/TNFα or TNFα alone (data not shown). This indicates that the overexpression of TIMP-1 or TIMP-3 did not induce a visible degree of apoptosis or cell death under our experimental conditions.

Inhibition of MT3-MMP reduces tube formation by hEMVEC

The inhibition of tube formation by both TIMP-1 and TIMP-3 overexpression indicates that other MMPs than MT1-MMP play a role in the regulation of tube formation by hEMVEC. To obtain evidence for the involvement of MT3-MMP in the regulation of this process, tube formation by hEMVEC was induced in the presence of anti-MT3-MMP IgG. Inhibition of MT3-MMP significantly reduced the VEGF-A-enhanced capillary-like tube formation by hEMVEC, while non-specific anti-FITC IgG had no effect (Figure 6). The inhibition of VEGF-enhanced tube formation by MT3-MMP IgG was 48.8% of the inhibition achieved by BB94, suggesting that other metalloproteinases may contribute additionally.

DISCUSSION

The present study demonstrates that both the u-PA/plasmin system and MMPs contribute to the invasion and tubular structure formation by endothelial cells in a 3D-fibrin-collagen matrix. Since TIMP-1 and TIMP-3 overexpression reduced capillary-like tube formation by hEMVEC to the same extent, not primarily MT1-MMP, but other MMPs play a regulatory role in this process in hEMVEC. Major MMPs expressed by hEMVEC were MMP-1, MMP-2, MT1-MMP (MMP-14) MT3-MMP (MMP-16) and MT4-MMP (MMP-17) under basal and VEGF-A-stimulated conditions. Our data suggest that MT3-MMP is in-

volved in the regulation of tube formation by hEMVEC, because tube formation by hEMVEC was inhibited of by anti-MT3-MMP IgG *in vitro*, and MT3-MMP was encountered in endothelial cells of proliferative endometrium *in vivo*.

Our data on the expression of MMPs by hEMVEC *in vitro* are in agreement with observations reported from immunohistochemical studies in endometrial tissue sections. Freitas *et al* found MMP-1, MMP-2, MMP-3 and MMP-9 in endometrial vascular structures, which might include endothelial cells, and MMP-2 was demonstrated in newly formed capillary strands [Freitas *et al.,* 1999]. Skinner *et al* only found MMP-9 on endometrial endothelial cells after exposure to high progestagen levels [Skinner *et al.,* 1999]. MT1-MMP was detected at low levels on endothelial cells in proliferative and secretory endometrium [Maatta *et al.,* 2000; Zhang *et al.,* 2000]. MT2-MMP was observed at a constant low level throughout the menstrual cycle [Goffin *et al.,* 2003; Zhang *et al.,* 2000]. In addition, TIMP-1, -2, and -3 were demonstrated in endometrial endothelial cells by *in situ* hybridization [Chegini *et al.,* 2003; Maatta *et al.,* 2000; Rodgers *et al.,* 1994]. Recently Goffin *et al* also reported the presence of MMP-19 mRNA in endometrial tissue throughout the cycle and the mRNAs of MMP-7, MMP-26 and MT3-MMP in this tissue during the proliferative phase of the cycle [Goffin *et al.,* 2003]. However, no information on their expression by specific cells is currently available.

Within the large group of MMPs the MT-MMPs attract specific attention, because of their membrane localisation that enables them to regulate localised proteolytic activities directly at the cell-matrix interaction sites. Hotary *et al* showed that overexpression of the transmembrane MT1-MMP, MT2-MMP or MT3-MMP induced endothelial invasion and tube formation in fibrin, while the GPI-anchored MT4-MMP was unable to do so [Hotary *et al.,* 2002]. MT1-MMP and MT3-MMP are involved in the migration and invasion of various mesenchymal cells, such as fibroblasts and smooth muscle cells [Vernon *et al.,* 1995], while other cells, such as leukocytes and trophoblasts, use MT2-MMP [Eeckhout, Vaes 1977; Giavazzi *et al.,* 2000]. Our data indicate that human endometrial endothelial cells *in vitro* largely express MT1-MMP, MT3-MMP and MT4-MMP while only tiny amounts of MT2- and MT5-MMP mRNA are present. Previous studies on HUVEC and hFMVEC indicated that invasion and tube formation of endothelial cells was inhibited by TIMP-3 and not by TIMP-1, suggesting that MT1-MMP has a dominant role among the MMPs in regulating endothelial migration and invasion [Anand-Apte *et al.,* 1997; Collen *et al.,* 2003; Galvez *et al.,* 2001; Hotary *et al.,* 2002; Lafleur *et al.,* 2002]. The present data confirm our previous data for hFMVEC, but also show consistently that both TIMP-1 and TIMP-3 inhibited tube formation by endometrial endothelial cells. Although these data do not exclude the involvement of MT1-MMP, they strongly suggest that other MMPs than MT1-MMP may contribute more dominantly to endometrial angiogenesis.

The expression of MMP-1 differed markedly between hEMVEC and hFMVEC, however a role for MMP-1 is less likely since MMP-1 is only upregulated in the secretory phase of the menstrual cycle and not in the proliferative phase. However, data on cell-specific expression are required before definitive conclusions can be drawn. A second possible explanation of the comparable inhibition by TIMP-1 and TIMP-3 might be that MT1-MMP acts in concert with other MMPs, in particular MMP-2, and that inhibition of the other MMPs is rate-limiting. However, the comparable expressions of MMP-2 and MT1-MMP in hEMVEC and hFMVEC do not favor this suggestion. Finally, a more likely candidate may be MT3-MMP, which like MT1-MMP can contribute potently to angiogenesis in a fibrinous matrix [Hotary *et al.,* 2002]. The recent finding that the expression of MT3-MMP mRNA is elevated in endometrial tissue during the proliferative phase of the menstrual cycle suggests such a role [Goffin *et al.,* 2003]. Furthermore, our data on the relative expressions of MT3-MMP mRNAs in hEMVEC and hFMVEC, the presence of MT3-MMP protein on endometrial endothelial cells and the inhibition of capillary tube formation by inhibiting MT3-MMP are strongly in favor of a contribution of MT3-MMP in capillary-like tube formation by hEMVEC.

To summarise, MMPs contribute to *in vitro* capillary tube formation by human endometrial endothelial cells. Whereas capillary tube formation by hFMVEC depends largely on MT1-MMP, the described data for hEMVEC suggest that other MMPs than MT1–MMP, in particular MT3-MMP, play an important role in tube formation by human endometrial endothelial cells.