Membrane-type matrix metalloproteinases and vascularization in human endometrium during the menstrual cycle

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Endometrial angiogenesis is essential for a vascularized receptive endometrium. Previously, we described that membrane type-3 metalloproteinase (MT3-MMP) is associated with endometrial angiogenesis *in vitro*. The association of MT-MMPs with endometrial angiogenesis *in vivo* is unknown. Therefore, this study analysed the presence of MT-MMPs in human endometrium and their correlation with neovascularization. RNA/protein expressions of the six MT-MMPs were determined in cultured endometrial cells. Vascularization parameters and MT-MMP expressions *in vivo* were evaluated by immunohistochemistry in serial endometrium sections. MT1-, MT2-, MT3- and MT4-MMP antigens were expressed in cultured endometrial endothelial cells. MT2-, MT3- and MT4-MMP were expressed by endothelium during the proliferative and secretory phase. Strikingly, these phases showed elevated vascularization, elevated total vascular surface in proliferative phases, elevated number of vessels in proliferative/late secretory phases and increased luminal surface in the proliferative phases. All MT-MMP antigens were expressed in various endometrial cell types *in vivo*, with decreased levels during the early secretory phase. In conclusion, all MT-MMPs are expressed in endometrium in a cycle-dependent pattern. The vascular expression of MT2-, MT3- and MT4-MMP correlated with angiogenic episodes of the cycle. Since MT2- and MT3-MMP are known to regulate tube formation, these findings support earlier *in vitro* data on the role of MT3-MMP in endometrial angiogenesis. Additionally, MT2-MMP appears to be associated with endometrial neovascularization also.

Key words: angiogenesis/endometrium/endothelium/MT3-MMP/MT-MMP

Introduction

Angiogenesis, the sprouting of new capillaries from existing vasculature, is essential to support endometrial reconstruction after menstruation and to provide a vascularized, receptive endometrium for implantation. Endometrial angiogenesis occurs periodically at three separate episodes during each cycle; during the early proliferative phase representing post-menstrual repair, during the mid-proliferative phase under the influence of oestrogen and during the secretory phase under the influence of mainly progesterone (Ferenczy *et al*., 1979; Goodger and Rogers, 1995; Rogers and Gargett, 1998; Smith, 2001). Most work to date concerning the regulation of endometrial angiogenesis has focused on steroidal hormones and vascular endothelial growth factor (VEGF). The steroidal hormones regulate endometrial growth and probably also endometrial angiogenesis either directly or indirectly through locally produced factors like VEGF and proteases (Shifren *et al*., 1996; Bausero *et al*., 1998; Iruela-Arispe *et al*., 1999; Perrot-Applanat *et al*., 2000).

Angiogenesis is facilitated by proteolysis, since endothelial cells require proteolytic activity to be able to degrade their basal membrane,

to migrate and to invade the underlying extracellular matrix (Heymans *et al*., 1999; Stetler-Stevenson, 1999; Pepper, 2001). Key regulators of proteolysis belong to the family of matrix metalloproteinases (MMPs) and in particular to the subgroup of membrane-type matrix metalloproteinases (MT-MMPs). Their membrane-associated localization makes them particularly suited to functioning in pericellular proteolysis (Hotary *et al*., 2000; Visse and Nagase, 2003). MT1-, MT2-, MT3- and MT5-MMPs are trans-membrane proteins, whereas MT4- and MT6-MMP are GPI anchored. MT-MMPs are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs); TIMP-1 inhibits all MT-MMPs, except MT1-MMP, while TIMP-2 and -3 inhibit all MT-MMPs (Hernandez-Barrantes *et al*., 2002; Visse and Nagase, 2003). MT1-MMP is also inhibited by TIMP-4 (Bigg *et al*., 2001).

Previous reports showed that MT-MMPs play an important role in angiogenesis (Hotary *et al*., 2000, 2002; Zhou *et al*., 2000). MT1-MMP has received considerable attention as being involved in tumour angiogenesis (Sounni *et al*., 2002; Seiki and Yana, 2003). Furthermore, MT1-, MT2- and MT3-MMP have been shown to be able to induce capillary-like tube formation *in vitro* (Hiraoka *et al*., 1998;

Hotary *et al*., 2000, 2002; Galvez *et al*., 2001; Lafleur *et al*., 2002; Collen *et al*., 2003). Capillary formation by human foreskin microvascular endothelial cells is mediated by MT1-MMP (Collen *et al*., 2003; Plaisier *et al*., 2004). However, we recently showed that capillary-like tube formation by human endometrial microvascular endothelial cells (hEMVEC) is mediated by MT3-MMP, suggesting that MT3-MMP may be a potential regulator of endometrial angiogenesis (Plaisier *et al*., 2004).

Whether MT-MMPs are associated with endothelial cells during endometrial angiogenesis *in vivo* is not clear. Furthermore, also the role of MT-MMPs in endometrial receptivity and remodelling is still indistinct. Several MT-MMPs have been demonstrated in whole endometrial extracts at mRNA level (Chung *et al*., 2002; Goffin *et al*., 2003), and MT1- and MT2-MMP antigens have been demonstrated in various endometrial cell types (Määttä *et al*., 2000; Zhang *et al*., 2000). However, little information is available regarding the expression of the six MT-MMPs in endothelium and their relationship to endometrial angiogenesis. Therefore, this study aims to analyse the expression of MT-MMPs by endometrial cells, including endothelium, to assess their endometrial expression during the menstrual cycle and to correlate these findings to the angiogenic status of the cycling endometrium.

Materials and methods

Collection of human endometrium samples
Endometrial samples were collected from 26 premenopausal and five postmenopausal women undergoing hysterectomy for benign gynaecological pathologies [leiomyoma ($n = 23$), uterine prolapse ($n = 1$), dysmenorrhoea ($n = 1$), menorrhagia ($n = 2$)]. Tissue was collected according to the guidelines of the institutional review board, and informed consent was obtained from each patient. None of the subjects had received steroid hormone therapy, i.e. OAC, IUD or HRT, for at least 3 months before surgery. The total number of donors with an irregular cycle was 4; $n = 1$ in early proliferative (EP), $n = 1$ in late proliferative (LP), $n = 1$ in early secretory (ES) and $n = 1$ in early secretory (LS) phase. Endometrial tissue (0.5–1.0 g) was scraped off the *fundus uteri*, using a surgical knife, immediately after surgery. When leiomyoma was present, tissue was obtained from the opposite side of the uterus. Tissue was either stored at 4°C or fixed in formaldehyde.

All premenopausal samples were dated by an independent pathologist using the endometrial dating criteria of Noyes *et al*. (1975). The histological appearance of the endometrium was classified as menstrual (M, day 28–3; $n = 2$), EP (day 4–10; $n = 5$), LP (day 11–14; $n = 7$), ES (day 15–20; $n = 6$) and LS (day 21–28; $n = 6$) phase. Samples were excluded when containing endometriosis, leiomyoma, adenomyosis or when dating was inconsistent with known last day of the menstrual cycle. The mean age of the premenopausal group was 42.4 years (range 31–48) and of the post-menopausal group 59.7 years (range 57–63). Age, pre-operative diagnosis and menstrual irregularity were equally distributed among the groups M, EP, LP, ES and LS.

Cell isolation and culture
hEMVEC, human endometrial microvascular epithelial (hEEC) and stromal (hESC) cells were isolated from premenopausal endometrium and cultured as previously described (Koolwijk *et al*., 2001). Cells were extracted from minced tissue using 0.2% collagenase and further separated using gravity sedimentation as follows. After setting for 5 min, the supernatant was separated from the epithelium-rich settled pellet. The heterogeneous supernatant, containing mainly stromal and endothelial cells, was further separated into hEMVEC and hESC by repeated selections with anti-CD31-coated Dynabeads (Dynal Biotech, Oslo, Norway). The pellet was resuspended in M199 with 10% newborn calf serum (NBCS) and placed in culture dishes. During the following 15 min, remaining stromal and endothelial cells attached to the dish, while epithelial structures remained in the media. By transferring the media and repeating this, an epithelial cell culture was derived. Cells were characterized by morphology and immunohistochemical markers; hEMVEC were positive for CD31 and von Willebrand factor and negative for cytokeratin-8 and -18.

hEEC were positive for cytokeratin-8 and -18, and hESC were negative for these markers.

hEMVEC were cultured in M199 with 20% human serum (HS), 10% NBCS, 37.5 µg/ml endothelial cell growth factor (ECGF), 5 U/ml heparin, 100 IU/ml penicillin and 100 mg/ml streptomycin. hESC and hEEC were cultured in M199 with 10% HS, 10% NBCS, 75 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. Human recombinant VEGF-A (5 ng/ml, RELIAtech, Braunschweig, Germany) was added to the culture of primary isolates of endothelial cells to facilitate initial growth.

Reverse transcription and real-time quantitative RT-PCR
Total RNA from cultured cells was isolated as described by Chomczynski and Sacchi (1987). RNA quantity and quality were determined in a spectrophotometer (Nanodrop ND-1000, OD_{260}/OD_{280} between 1.8 and 2.0). Reverse transcription was performed with 1 µg total RNA, random primers and a cDNA synthesis kit (Promega Corporations, Madison, WI, USA).

RNA expression was quantified according to the Taqman real-time PCR method (Applied Biosystems, Foster City, CA, USA, Perkin Elmer, Wellesley, MA, USA) using primers combined with a specific (FAM/TAMRA) double-labelled probe. Sequences of the primers and probes used to detect MT-MMPs were described previously (Collen *et al*., 2003; Plaisier *et al*., 2004). The endogenous reference gene GAPDH (primers/VIC-labelled probe, Applied Biosystems) was used to correct for the total mRNA content of the samples. Other genes, β-actin and cyclophilin, were also used as reference genes and showed comparable results (data not shown). The primer efficiencies were verified by determining a standard curve using serial dilutions of hESC cDNA. Primers were considered efficient when the calculated efficiency ranged between 90 and 110%. RT-PCR reactions for MT-MMP/GAPDH pairs were performed in duplicate and expressed in cycle times (Ct), the number of cycles required for the PCR reaction to enter the logarithmic phase. The difference in Ct values (dCt) was calculated as the difference in Ct of the target gene and the reference gene GAPDH. Water and '–RT samples', obtained by the omission of the RT enzyme in the cDNA reaction, were used as negative controls.

Immunohistochemistry on cultured cells
Cultured cells were fixed for 10 min with cold 2% *p*-formaldehyde followed by 1 min with cold acetone. After three washes with PBS, the cells were incubated for 15 min with 5% HS and 5% goat serum in PBS to reduce aspecific binding. Subsequently, the cells were incubated with a first antibody overnight at 4°C. The following commercially available first antibodies were used: monoclonal mouse anti-MT2-MMP, monoclonal mouse anti-MT3-MMP (162-22G5 and 117-10C6, Oncogene, San Diego, CA, USA), polyclonal rabbit anti-MT4-MMP, polyclonal rabbit anti-MT5-MMP (M3684 and M6684, Sigma, St Louis, MO, USA) and polyclonal rabbit anti-von Willebrand factor (A 0082, Dakocytomation, Glostrup, Denmark). Anti-MT1-MMP antibodies were produced and characterized as previously described (Van Boheemen *et al*., 1995; Koolwijk *et al*., 1996; Collen *et al*., 2003; Plaisier *et al*., 2004), and polyclonal goat anti-MT6-MMP was a generous gift from W. English (Cambridge, UK). After three washes with PBS, the cells were incubated with an FITC-labelled secondary antibody for 1 h at room temperature and finally washed three times with PBS. The DAPI (Vector laboratories, Burlingame, CA, USA) was added, and cells were mounted and evaluated using a Zeiss Axiovert 200 M inverted microscope. To detect lysosomes, an acidic organelle-selective probe was used, which accumulates in lysosomes and is linked to a fluorescent label. This Lysotracker Red DND-991 µM (L-7528, Molecular Probes by Invitrogen, Breda, Netherlands) was added to unfixed cultured cells and visualized.

Immunohistochemistry on paraffin sections
Tissue samples were processed into paraffin sections as previously described (Kroon *et al*., 1999). Besides the above-described first antibodies, monoclonal mouse CD34 (QBend/10, Novocastra, Newcastle upon Tyne, UK) was also used on the tissue samples. Endogenous peroxidase was quenched with 3% H_2O_2/methanol, and aspecific binding was reduced by incubation with 5% bovine serum albumin. Antigen retrieval in trypsin was used for the detection of MT2- and MT4-MMP. Primary antibodies were applied overnight at 4°C followed by incubation with a biotinylated secondary antibody. Antibody binding was visualized using avidin–biotin and streptavidin–horseradish peroxidase

conjugates. Sections were counterstained with Mayer's hematoxylin. Specificity of the reaction was verified by the omission of the first antibody or using mouse, goat or rabbit serum as first antibody.

Evaluation of the vascularization pattern

CD34-stained sections were used to scan six fields, randomly taken throughout the stained sections, at ×100 magnification. CD34-positive vessels were counted, and their luminal surface was measured using image analysis software (Qwin) developed by Leica Microsystems. The total vascular surface (mm^2/mm^2), the number of vessels per mm^2 and the luminal surface ($\mu m^2/vessel$) were calculated using the same software.

Evaluation of immunohistochemical staining

Immunostaining in all samples was evaluated at ×100 magnification, by calculating a staining index (SI) according to the following equation: proportion of stained cells × staining intensity. The proportion of stained cells was expressed as 0, 1, 2 or 3 (positive staining signal in 0, <10, 10–50 or >50% of cells, respectively) and intensity of staining as 1, 2 or 3 (weak, moderate or strong staining, respectively). The minimum score was 0 and the maximum score was 9 (Nap *et al.*, 2004). The average of the scores of two independent observers was used to calculate the mean SI, which represents the protein expression per cell type, namely glandular epithelial cells, stromal cells, endothelial cells and perivascular smooth muscle cells (PSMC). The inter-observer variability was 4% for MT1-MMP, 14% for MT2-MMP, 8% for MT3-MMP, 10% for MT4-MMP, 9% for MT5-MMP and 5% for MT6-MMP (mean variability 8%). Total mean SI were calculated as the sum of mean staining indices in each tissue sample and represent the total protein expression per tissue.

Statistical analysis

The one-way analysis of variance (ANOVA) and the Bonferroni post-hoc test were used to examine the MT-MMP expression levels in endometrial cells *in vitro*. The Kruskal–Wallis test, a nonparametrical test for more than two independent samples, was used to examine the differences between the stages of the menstrual cycle. Results were considered statistically significant when $P < 0.05$ (SPSS, version 11.5).

Results

Vascularization pattern in endometrium during the cycle

Analysis of the endometrial vascularization parameters, using CD34-stained paraffin sections (Fig. 1A), showed that the total vascular surface was elevated in the late proliferative phase, $0.05 \ mm^2/mm^2$ compared to the other phases ($P < 0.01$, Fig. 1B). Vessel density ranged between 132 and 195 vessels per mm^2 (mean $168/mm^2$) during the cycle. LP, LS and M endometrium showed an increased number of vessels compared to EP and ES ($P < 0.02$, Fig. 1C). Finally, the luminal surface was raised in the first half of the cycle, 331–398 $\mu m^2/vessel$, compared to the second half, 178–193 $\mu m^2/vessel$ ($P < 0.05$, Fig. 1D).

MT-MMP expression in endometrial cells **in vitro**

To screen which MT-MMPs are expressed by endometrial cells, we first determined the expression of the MT-MMPs by hEMVEC, hESC and hEEC *in vitro* using RT-PCR. hEMVEC mainly expressed MT1-,
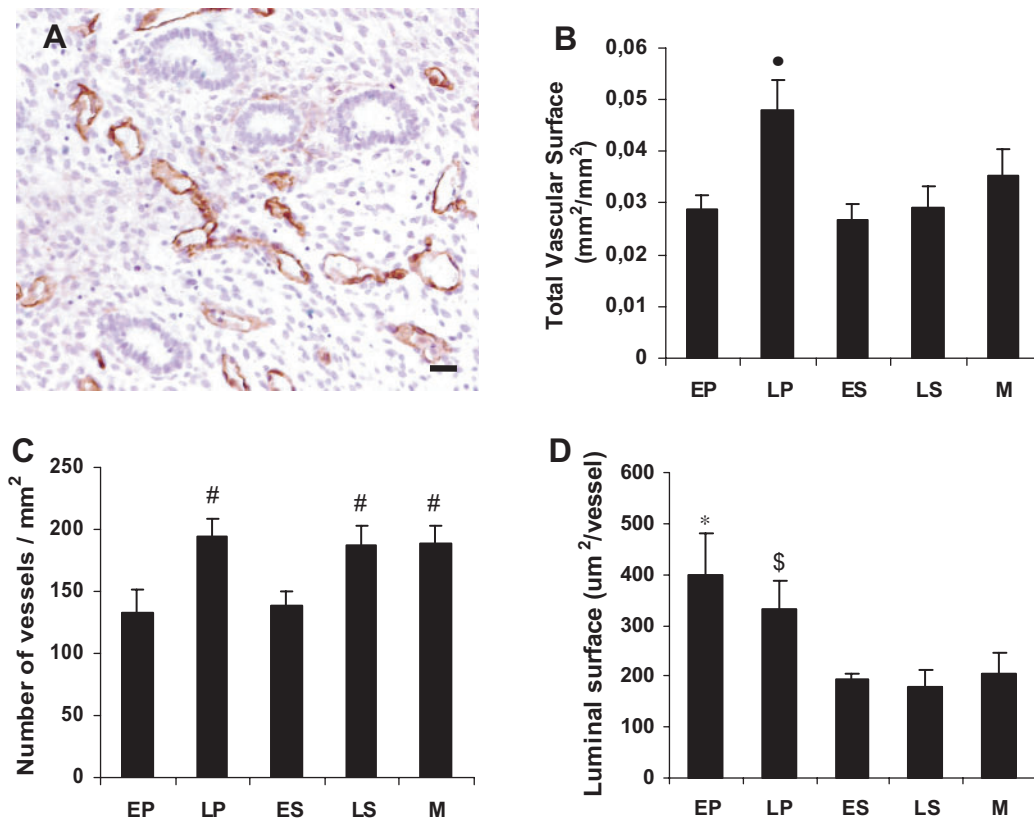
Figure 1. Vascularization pattern in cycling human endometrium. The vascularization pattern in human endometrium was determined by image analysis of anti-CD34-stained sections. Six fields per biopsy were scanned at ×100 magnification. (**A**) CD34 antigen expression in endothelial cells in late proliferative endometrium (bar = 50 μm), (**B**) The total vascular surface (mm^2/mm^2), (**C**) the number of vessels per mm^2 and (**D**) the luminal surface ($\mu m^2/vessel$) were calculated for each sample and expressed as mean ± SEM. EP, early proliferative phase ($n = 6$); LP, late proliferative phase ($n = 7$); ES, early secretory phase ($n = 6$); LS, late secretory phase ($n = 6$); M, menstrual phase ($n = 2$); •$P < 0.01$ versus EP, ES and LS; #$P < 0.02$ versus EP, ES; *$P < 0.01$ versus ES, LS and M; and \$$P < 0.05$ versus ES, LS and M.

MT3- and MT4-MMP mRNAs (dCt < 9.0, Table I), whereas MT2-, MT5- and MT6-MMP mRNA were weakly expressed (Table I).

Strikingly, MT1-MMP mRNA expression was considerably lower in endothelial cells compared to both stromal and epithelial cells (ΔdCt 6.1 and 6.1, $P < 0.01$). Epithelial cells expressed most MT-MMPs more abundantly than the other cell types ($P < 0.01$, Table I).

At the protein level, all MT-MMPs were expressed by hEMVEC but were rather located in the cell rather than on the cell membrane (Fig. 2). MT1-, MT2-, MT3- and MT4-MMP were clearly and consistently detectable (Fig. 2A–G), whereas MT5- and MT6-MMP antigens were only weakly present (data not shown). The MT-MMPs showed a perinuclear staining (compare its localization with the cortical F-actin of the cells in Fig. 2B,D,F), often in a granular pattern. This granular pattern was distinct from that of von Willebrand factor in Weibel Palade bodies (Fig. 2H) but, in part, resembled the lysosomal compartment as visualized by lysotracker, an

Table I. Membrane-type metalloproteinase (MT-MMP) mRNA expression in human endometrial cells *in vitro*

	hEMVEC		hESC		hEEC	
	Ct	dCt	Ct	dCt	Ct	dCt
MT1-MMP	27.8 ± 0.4	9.0 ± 0.4	22.8 ± 1.5	2.9 ± 1.5*	22.7 ± 0.5	2.9 ± 0.1*
MT2-MMP	33.7 ± 1.2	14.1 ± 1.2	35.6 ± 1.8	15.6 ± 0.6	29.0 ± 0.3	9.2 ± 0.3*
MT3-MMP	26.4 ± 0.2	7.4 ± 0.4	30.5 ± 0.4	10.6 ± 1.2*	25.0 ± 0.6	6.3 ± 0.4
MT4-MMP	26.9 ± 0.2	7.3 ± 0.3	28.3 ± 1.1	9.0 ± 0.4*	29.8 ± 0.1	10.1 ± 0.1*
MT5-MMP	31.6 ± 0.3	12.8 ± 0.3	33.5 ± 1.4	14.6 ± 0.5*	27.7 ± 0.6	7.9 ± 0.4*
MT6-MMP	36.3 ± 1.0	17.7 ± 0.8	35.2 ± 1.6	14.4 ± 2.5	32.4 ± 0.6	12.2 ± 0.7

RNA was isolated from cultured hEMVEC (three donors), hESC (five donors) and hEEC (three donors), and cDNA was synthesized as described. Donors were premenopausal and of proliferative phases. RT-PCR for MT-MMP/GAPDH pairs was performed in duplicate and expressed as the number of cycles (Ct ± SEM). GAPDH was used to correct for the total mRNA content of the samples (mean GAPDH Ct 19.3 ± 0.3). The dCt values were calculated as the difference in the number of cycles required for the PCR reaction of the target gene and of the reference gene to enter the logarithmic phase and expressed as dCt ± SEM. hEMVEC, human endometrial microvascular endothelial cells; hEEC, human endometrial microvascular epithelial cells; hESC human endometrial microvascular stromal cells.
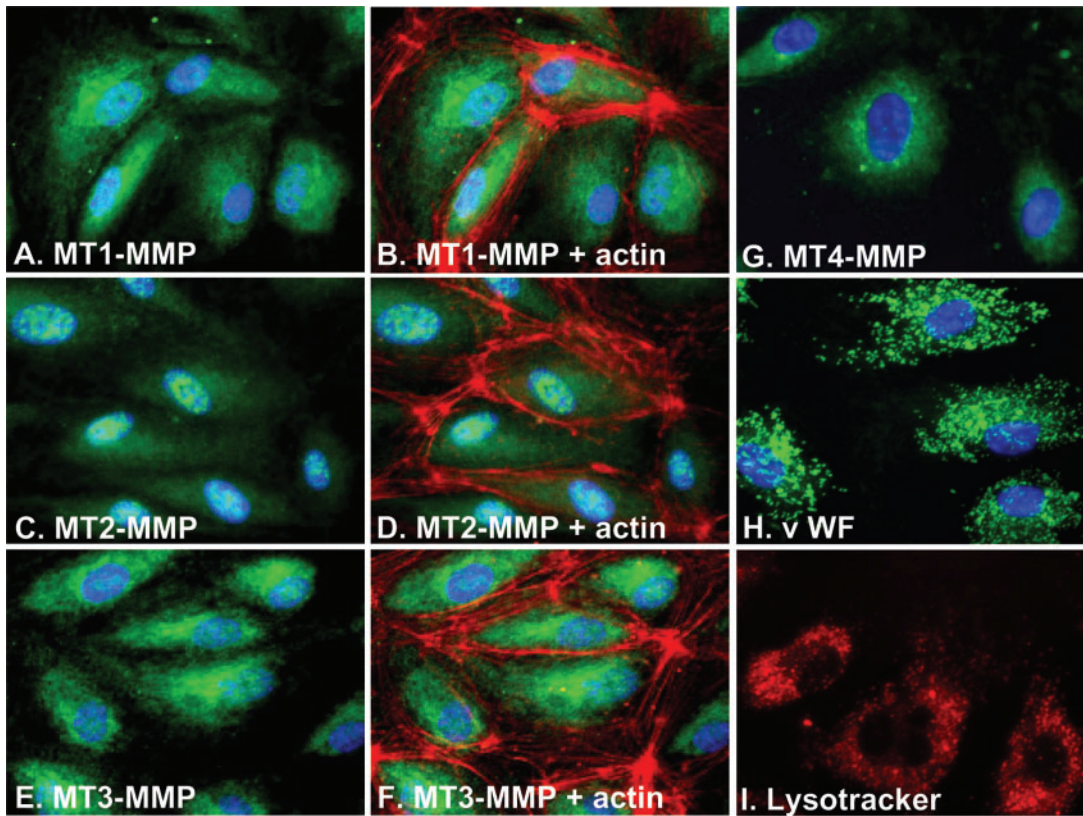*$P < 0.01$ versus hEMVEC.

Figure 2. Membrane-type metalloproteinase (MT-MMP) antigen expressions in human endometrial endothelial cells (hEMVEC). Endothelial MT-MMP expression was determined by fluorescence microscopy (magnification ×40). The expression of MT1-MMP (± actin, **A**, **B**), MT2-MMP (± actin, **C**, **D**), MT3-MMP (± actin, **E**, **F**), MT4-MMP (**G**), von Willebrand Factor in Weibel Palade bodies (**H**) and Lysotracker in lysosomes (**I**) in cultured hEMVEC. The negative control only showed blue staining of the nuclei (not shown). Blue, DAPI in nuclei; green, target protein; red, actin or lysotracker.

acidic organelle-selective fluorescent probe (Fig. 2I). Unexpectedly, MT2-MMP antigen was also encountered in the nuclei.

MT-MMP antigen expressions in cycling human endometrium

Subsequently, we investigated the presence of the six MT-MMP antigens in cycling endometrium by immunohistochemistry in serial

paraffin sections of the samples that were also used for the evaluation of the vascularization pattern.

MT-MMP antigen expressions in vascular cells

Endometrial endothelium exhibited weak expression of MT-MMP antigens with a maximal SI of 2.2 (Fig. 3A, Table II). MT2-MMP was most abundantly expressed by endothelium during the EP, LP and LS phase,
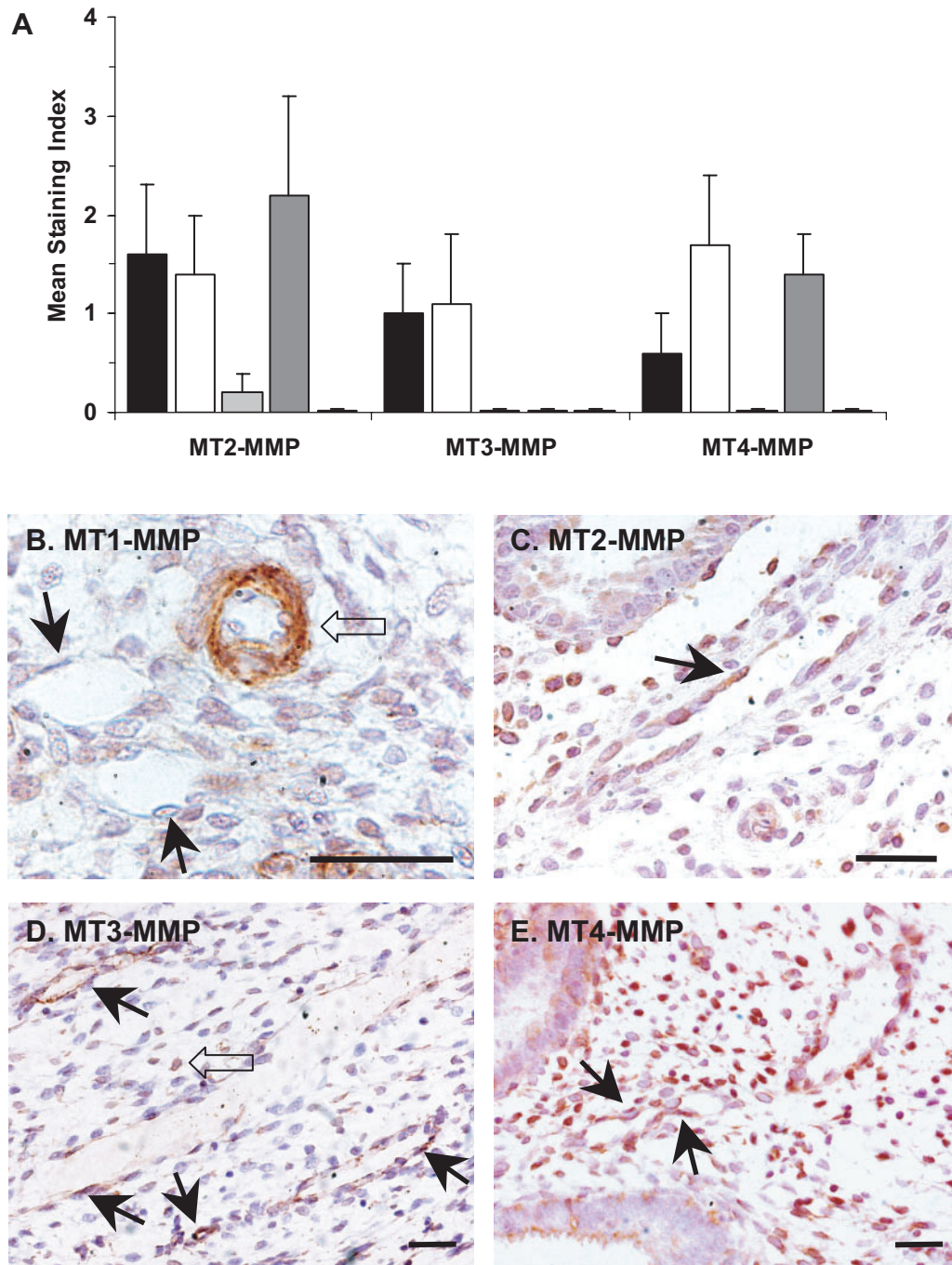
Figure 3. Membrane-type metalloproteinase (MT-MMP) antigen expressions in endometrial endothelium and other cell types. The expression of MT-MMP antigens by endometrial endothelial cells was determined by immunohistochemistry. (A) MT-MMP antigen expressions in endothelial cells expressed per cycle phase as mean staining index ± SEM (all $P > 0.05$). The staining index is minimally 0 (no stained cells) and maximally 9 (>50% strongly stained cells). (B) MT1-MMP antigen detected in perivascular smooth muscle cells (open arrow) but not in endothelial cells (arrows) in late proliferative (LP) endometrium. (C) MT2-MMP antigen expression in endothelium in LP endometrium (arrow). (D) MT3-MMP expression in endothelial cells (arrows) and stromal cells (open arrow) in LP endometrium. (E) MT4-MMP expression in endothelial cells (arrow) in LP endometrium. (F) negative control. Bar = 50 μm. ■ = early proliferative phase ($n = 6$), □ = late proliferative phase ($n = 7$), ▨ = early secretory phase ($n = 6$), ▨ = late secretory phase ($n = 6$), ▨ = menstrual phase ($n = 6$).

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Table II. Membrane-type metalloproteinase (MT-MMP) antigen expression during the menstrual cycle

	EC	PSMC	GE	SC	Total SI
MT1-MMP					
EP	0.0	4.1	5.2	0.6	9.9
LP	0.0	5.0	5.0	1.3	11.3
ES	0.0	4.2	4.8	2.2	11.2
LS	0.0	5.6	4.0	1.8	11.4
M	0.0	4.5	4.5	0.0	9.0
MT2-MMP					
EP	1.6	7.0	4.8	5.2	18.6
LP	1.6	5.7	7.3	4.9	19.6
ES	0.2	5.8	4.4	3.6	14.0*
LS	2.2	6.2	5.0	5.8	19.2
M	0.0	2.0	1.5	1.5	5.0
MT3-MMP					
EP	1.0	5.3	4.4	2.2	12.9
LP	1.0	4.6	3.6	1.9	11.0
ES	0.0	3.0	1.3	1.3	5.5**
LS	0.0	4.0	3.4	1.8	9.2
M	0.0	3.0	5.0	0.0	8.0
MT4-MMP					
EP	0.6	5.0	3.6	1.8	11.0
LP	1.7	4.9	5.3	3.7	15.6
ES	0.0	5.0	5.3	1.3	11.5*
LS	1.4	7.8	4.2	5.4	18.8
M	0.0	2.5	1.5	1.5	5.5
MT5-MMP					
EP	0.0	1.0	1.8	0.2	3.0
LP	0.0	4.7	3.1	0.7	8.6
ES	0.0	3.0	3.5	0.3	6.8
LS	0.0	3.6	3.0	0.8	7.4
M	0.0	4.0	3.5	1.5	9.0
MT6-MMP					
EP	0.0	3.0	2.0	0.5	5.5
LP	0.0	3.9	3.9	1.0	8.7
ES	0.0	3.0	3.0	0.8	6.8***
LS	0.0	5.0	3.0	2.3	10.3
M	0.0	0.0	3.0	0.0	3.0

The MT-MMP antigen expressions are expressed as the mean staining indices per cell type per phase, which is minimally 0 (no stained cells) and maximally 9 (>50% strongly stained cells). The total staining indices (total SI) per phase is calculated as the sum of the mean SI of endothelial cells (EC), perivascular smooth muscle cells (PSMC), glandular epithelium (GE) and stromal cells (SC). EP, early proliferative (*n* = 6); LP, late proliferative (*n* = 7); ES, early secretory (*n* = 6); LS, late secretory (*n* = 6); M = menstruation (*n* = 2).
*$P < 0.05$ versus LP and LS.
**$P < 0.05$ versus EP and LP.
***$P < 0.05$ versus LS.

in a similar way to MT4-MMP ($P > 0.05$, Fig. 3A,C,E). Vascular MT3-MMP antigens were only detected during the EP and LP phase (Fig. 3A,D). MT5- and MT6-MMP antigens were not detected in endothelium. Surprisingly, MT1-MMP was also not detected in endothelium, while it was clearly present in other cell types (Fig. 3B). MT1-MMP was also clearly present in cervix carcinoma endothelium in a parallel experiment (data not shown).

PSMC expressed all MT-MMPs abundantly throughout the cycle, with a mean SI of 4.2 (range 1.0–7.9, Table II, Fig. 3B).

MT-MMP antigen expressions in endometrial cells
other than vascular cells
All MT-MMPs were expressed by epithelial and to a lesser extent by stromal cells (Table II). Both cell types expressed higher amounts of MT1-, MT2-, MT3- and MT4-MMP than that of MT5- and MT6-MMP. All MT-MMPs were highly expressed in the smooth muscle cells of the myometrium (data not shown).

MT-MMP regulation during the menstrual cycle and the post-menopause

MT-MMP antigen expressions during the menstrual cycle
First, total MT-MMP antigen expressions in proliferative phase were compared to those in the ES phase, showing that MT2-, MT3- and MT4-MMP expressions were markedly lower in ES endometrium (total SI differences 5.3, 8.8 and 4.3 respectively, $P < 0.05$, Table II). The expression of MT1-, MT5- and MT6-MMP only slightly decreased in the ES phase compared to the proliferative phases (Table II).

Subsequently, ES phase was compared to the LS phase, demonstrating that total MT2-, MT3-, MT4- and MT6-MMP expressions were elevated during the LS phase compared to the ES phases (MT2-, MT4- and MT6-MMP $P < 0.05$, MT3-MMP $P > 0.05$, Table II). The total SI of MT1-and MT5-MMP remained rather constant during the cycle phases (Table II).

All MT-MMPs were expressed in considerable amounts during the menstrual phase. Unfortunately only two biopsies of menstrual phase endometrium were available, and therefore the staining patterns can only be indicative.

MT-MMP antigen expressions in post-menopausal
endometrium
Post-menopausal endometrial biopsies showed less abundant amounts of MT-MMP antigens as compared with the MT-MMP expressions in premenopausal endometrium. Nevertheless, all MT-MMPs, except MT5-MMP, were present in post-menopausal endometrium. In post-menopausal tissues, the total SI of MT1-MMP was 4.3, that of MT2-MMP was 4.5, that of MT3-MMP 3.3, that of MT4-MMP 3.0, that of MT5-MMP 0.0 and that of MT6-MMP was 1.0. Post-menopausal MT-MMPs were mainly expressed by epithelial cells and not by endothelium or PSMC.

Discussion

The present study showed that all MT-MMPs are expressed in human endometrium, of which most expressions show a cycle-dependent pattern. Furthermore, vascular MT2-, MT3- and MT4-MMP expression correlates with angiogenic episodes of the menstrual cycle. Since MT2- and MT3-MMP are known stimulators of angiogenesis *in vitro*, these findings suggest that MT2- and MT3-MMPs are associated with endometrial angiogenesis (Hotary *et al*., 2000; Plaisier *et al*., 2004).

MT-MMPs act at the cell surface where they can locally facilitate degradation of extracellular matrix, cell migration, invasion and angiogenesis. The abundance of all MT-MMP in cycling endometrium and the relatively weak MT-MMP expressions in the post-menopausal endometrium suggest that endometrial MT-MMPs play a role in remodelling of cycling endometrium.

A possible function of MT-MMPs may be associated with the shedding process, since their antigens were highly expressed during the LS and M phase and other MMPs have been shown to be associated with menstruation as well (Salamonsen and Woolley, 1996; Lockwood *et al*., 1998; Zhang *et al*., 2000). Alternatively, MT-MMPs may play a role in endometrial remodelling in preparation for implantation. Most MT-MMP expressions were reduced during the receptive window, probably under the influence of steroidal hormones. The low amount of MT-MMPs during the generation of receptive endometrium may be essential for the establishment of a stable environment in preparation for embryonic implantation. However, whether MT-MMPs play a role during implantation remains as yet unresolved, and this should be assessed in first-trimester decidua.

Another function of MT-MMPs may be the regulation of angiogenesis. Assuming the participation of MT-MMPs in angiogenesis, their

antigens must be expressed by endothelium during phases of the menstrual cycle with high angiogenic activity. Therefore, the present study did not only analyse the expression of MT-MMPs in cultured endometrial endothelial cells and in endometrial tissue samples but also determined their association with vascularization in these samples.

MT2-, MT3- and MT4-MMP were expressed by both *in vitro* and *in vivo* endothelial cells. Endometrial endothelium *in vivo* expressed MT3-MMP during the proliferative phase, whereas MT2- and MT4-MMP were expressed during both proliferative and LS phases. Strikingly, the endothelial MT-MMP expressions were expressed during phases with high angiogenic activity. The proliferative phase, and to a lesser extent the LS phase, showed an elevated endometrial angiogenesis, since respectively three and one vascularization parameter(s) were raised in these phases. Previous studies have described these angiogenic episodes as well (Ferenczy *et al*., 1979; Goodger and Rogers, 1995; Morgan *et al*., 1996; Rogers and Gargett, 1998; Smith, 2001).

MT1-MMP is expressed by endothelium *in vitro*, whereas its endothelial expression *in vivo* was consistently not detectable. This difference may be explained by translational regulation or culture-induced changes in *in vitro* cell metabolism. MT1-MMP expression is regulated by several signal transduction pathways, including the MAPK and PI3K pathways, which are influenced by cytokines and growth factors (Chakraborti *et al*., 2003; Boyd *et al*., 2004; Galvez *et al*., 2005). Since HS, ECGF (containing acidic FGF) and VEGF were used in the culture system, signalling pathways could have been activated and influenced MT1-MMP expression by endometrial cells (Plaisier *et al*., 2004).

The endothelial expression of MT2-MMP in both phases and the consistent absence of endothelial MT1-MMP is in accordance with previous work by Zhang *et al*. (2000). They showed that MT1-MMP antigens were present in all endometrial cell types but were 'virtually absent' in endothelial cells. Määttä *et al*. (2000) detected weak MT1-MMP mRNA expression in endothelium but did not study MT1-MMP protein expression. No data regarding the cellular expression of MT3- and MT4-MMP have been reported.

Although MT1-MMP is generally accepted to contribute to angiogenesis, MT2- and MT3-MMP are also described as stimulators of angiogenesis *in vitro* (Hotary *et al*., 2000, 2002; Zhou *et al*., 2000; Galvez *et al*., 2001; Lafleur *et al*., 2002). MT4-MMP is not able to induce capillary tube formation in 3D matrices *in vitro* (Hotary *et al*., 2002). In a recent *in vitro* study regarding endometrial angiogenesis, we showed that capillary-like tube formation by endometrial endothelial cells is not predominantly regulated by MT1-MMP but rather by MT3-MMP (Plaisier *et al*., 2004). These *in vitro* findings are supported by the absence of endothelial MT1-MMP *in vivo* and the presence of MT3-MMP antigens in endothelium *in vivo* during the angiogenic proliferative phase. Moreover, the detection of endothelial MT2-MMP expression during the angiogenic proliferative and LS phase suggests MT2-MMP to be an additional regulator of angiogenesis to consider (Hiraoka *et al*., 1998; Hotary *et al*., 2000; Lafleur *et al*., 2002). Future research should clarify their involvement in endometrial angiogenesis and the role of MT4-MMP in endothelium.

A remark should be made about the analysed endometrium samples, which were mainly obtained from women diagnosed with leiomyoma. Although the samples were obtained distant of the lesions and were microscopically 'normal', it is noteworthy that recently a relation between MMPs and myoma has been described. An altered level of MMP-9 was found in uterine cavities of leiomyoma patients (Inagaki *et al*., 2003), but whether this, or myoma themselves, can affect MT-MMP production by endothelial or other endometrial cells remains unclear.

The present study showed that all MT-MMPs were expressed at the protein level in human endometrium in a cycle-dependent pattern, with decreased expression levels in hormone-dependent secretory phases.

Furthermore, the expression of MT2- MT3- and MT4-MMP antigens by endometrial endothelium correlated with angiogenic episodes of the menstrual cycle. Since MT2- and MT3-MMP are known to regulate capillary-like tube formation, these findings support earlier *in vitro* data on the role of MT3-MMP in the regulation of endometrial angiogenesis. In addition to MT3-MMP, MT2-MMP may be associated with vascular remodelling processes in cycling endometrium as well.

Acknowledgements

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