Pericellular-acting proteases in human first trimester decidua

Margreet Plaisier\(^{1,2}\), Pieter Koolwijk\(^{2,3}\), Florian Willems\(^4\), Frans M. Helmerhorst\(^{1,5}\) and Victor W.M. van Hinsbergh\(^3\)

\(^{1}\)Division of Reproductive Medicine, Department of Gynaecology, Leiden University Medical Centre, PO Box 9600, 2300 RC, Leiden, The Netherlands; \(^{2}\)Gaubius Laboratory TNO-QoL, Department Biomedical Research, Zernikedreef 9, 2333 CK, Leiden, The Netherlands; \(^{3}\)Department of Physiology, Institute for Cardiovascular Research, VU Medical Centre, van der Boechorststraat 7, 1081 BT, Amsterdam, The Netherlands; \(^{4}\)ST TIMEZO Plus Clinic, van Beverninkstraat 134, 2582 VL, The Haque, The Netherlands

Correspondence address. Fax: +31-71-5248181; E-mail: M.Plaisier@lumc.nl, F.M.Helmerhorst@lumc.nl

Proteolysis is essential for decidual development during embryonic implantation, but little is known regarding the expression and functions of membrane-type matrix metalloproteinases (MT-MMPs) and urokinase-type plasminogen activator (uPA) and its receptor uPAR in decidua. Therefore, their protein and mRNA levels were analysed in three first trimester decidual tissues, decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB). Decidua was obtained during first trimester pregnancy termination. uPA, uPAR, and MT1/2/3/5-MMP expression were studied by RT-PCR and immunohistochemistry, and CD56-positive uNK cells and CD68-positive macrophages were quantified in serial sections. The mRNAs and antigens of all proteases and uPAR were detectable in the decidual tissues and extravillous trophoblasts (EVT). mRNA levels of all proteases and uPAR, except MT5-MMP, were elevated in both DB and DP compared to DSE, being significant for MT1-MMP and uPAR in DP. MT2- and MT3-MMP mRNAs in DB were 24- and 10-fold higher than in DSE, and 19- and 7-fold increased compared to DP. At the protein level uPA and uPAR were particularly elevated in DB, while pro-angiogenic MT1- and MT3-MMPs were elevated in both DB and DP compared to DSE. MT2-MMP was prominently present in all conditions. The number of uNK cells was increased in DB and DP versus DSE, while a comparable increase in macrophages did not reach statistical significance. These data are consistent with a differential regulation of pericellular proteases in decidua by pregnancy-induced hormones, immune cells and EVT.

**Keywords:** decidua; first trimester; matrix metalloproteinases; trophoblast; uNK cells

**Introduction**

Proteolysis is essential for decidual remodelling and vascularization during the inception of pregnancy. Disturbances in decidual development may play a role in the pathogenesis of spontaneous abortions and pre-eclampsia (Vailhe et al., 1999; Vuorela et al., 2000; Solberg et al., 2003; Zygmunts et al., 2003). Endometrial adaptation to fertilization starts during the receptive secretory phase of the menstrual cycle and continues throughout the first trimester. This adaptation includes decidualization, tissue remodelling, angiogenesis, and immune cell invasion. The invasion of immune cells is enormous: from 8% of total stromal cells (SC) during the menstrual cycle up to 30% during the first trimester. Approximately 70% of these leucocytes are uterine natural killer (uNK) cells and 10% are macrophages (Balmer et al., 1991).

Decidual remodelling, cell invasion as well as angiogenesis are facilitated by proteolysis. Trophoblasts, uNK cells and endothelial cells (EC) require proteolytic activity to degrade their extracellular matrix (ECM) proteins and to migrate (Heymans et al., 1999; Salamonsen 1999; Stetler-Stevenson 1999; Al-Atrash et al., 2001; Pepper 2001). These cells are able to generate proteolytic activity by either producing proteases or using proteases on neighbouring cells and/or ECM (Salamonsen 1999; Albertsson et al., 2000; Kim et al., 2000; Koolwijk et al., 2001; van den Heuvel et al., 2005). Key regulators of proteolysis belong to the family of metalloproteinases (MMPs), in particular to the subgroup of membrane-type MMPs (MT-MMPs), and to the plasmin/plasminogen system (Reuning et al., 2003; Kindzelskii et al., 2004; Alfano et al., 2005).

The membrane-associated localization of MT-MMPs makes them suited for pericellular proteolysis (Hotary et al., 2000; Egeblad and Werb, 2002; Seiki, Yana 2003; van Hinsbergh et al., 2006). We studied the transmembrane-spanning MT-MMPs, MT1-(MMMP-14), MT2- (MMMP-15), MT3- (MMMP-16) and MT5-MMP (MMMP-24). Only these MT-MMPs have the proteolytic potential to induce capillary tube formation, whereas the GPI-anchored MT4- and MT6-MMPs were unable to do so (Hotary et al., 2000). MT-MMPs are inhibited by tissue inhibitors of MMPs (TIMPs); TIMP-1 inhibits all MT-MMPs, except MT1-MMP, whereas 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).

MT1-MMP is the best known MT-MMP, which degrades ECM components and promotes cell migration, angiogenesis and tumour metastasis (Hiraoka et al., 1998; Galvez et al., 2001; Lafleur et al., 2002; Sounni et al., 2002; Collen et al., 2003; Seiki and Yana, 2003; Visse and Nagase, 2003). MT2- and MT3-MMP are less well studied and are known to be involved in cell migration and invasion. MT1-, MT2- and MT3-MMP induce angiogenesis in vitro and...
MT2- and MT3-MMP may even be potential regulators of endometrial angiogenesis in vivo (Hotary et al., 2000, 2002; Lafleur et al., 2002; Hiraoaka et al., 1998; Galvez et al., 2001; Collen et al., 2003; Plaisier et al., 2004; Plaisier et al., 2006). MT5-MMP is known for a gelatinolytic effect in the brain, which induces embryonic brain development and axonal growth (Llano et al., 1999; Pei, 1999).

With regard to MT-MMPs in decidua, only MT1- and MT2-MMP have been studied. MT1- and MT2-MMP RNA and protein expression are described in decidual extracts, SC, and the extra-villous trophoblast (EVT) (Hurskainen et al., 1998; Nawrocki et al., 1996; Bjorn et al., 2000; Nakano et al., 2001; Curry and Osteen, 2003; Bai et al., 2005). MT-MMPs are assumed to regulate trophoblast invasion during implantation (Salamonsen, 1999). Whether migration of other cell types, e.g. immune and EC, is also regulated by MT-MMPs remains to be seen.

The plasminogen activator (PA) system is based on the protease plasmin, which cleaves most ECM components. The circulating protein plasminogen is converted into the active protease plasmin by either tissue-type PA (tPA) or urokinase-type PA (uPA). tPA is mainly involved in clot dissolution, whereas uPA mediates pericellular proteolysis during cell migration, tissue remodelling and angiogenesis (Van Hinsbergh et al., 2006). uPA binds a specific cell-surface receptor, uPAR, which restricts the uPA-activity to the cell environment and enables activation of plasmin directly on the cell surface. The activity of uPA is regulated by at least two specific serine proteinase inhibitors, PA inhibitor type-1 and -2 (PAI-1/2) (Spengers and Kluft, 1987).

The role of uPA mediated plasminogen activation in cell migration has been studied for a variety of cells and for EC, leucocytes and trophoblasts in particular (Blasi et al., 1987; Heymans et al., 1999; Hu et al., 1999; Pepper, 2001; Reuning et al., 2003; Salamonsen et al., 2003). Both uPA and uPAR expression has been detected in the invasive trophoblast cells, which indicates a role for uPA and uPAR in trophoblast invasion (Hofmann et al., 1994; Hu et al., 1999; Multhaupt et al., 1994; Pierleoni et al., 1998; Salamonsen et al., 1999).

Little information is available regarding the expression of MT-MMPs and uPA/uPAR in the various decidual tissues and cell types. Moreover, their regulation and involvement in decidual remodelling, vascularization and immune cell and trophoblast invasion is not well established.

Endometrial adaptation to pregnancy is induced by pregnancy-induced hormones, i.e. hCG, estradiol (E2) and progesterone, immune cells and the EVT. The differential presence of these factors results in the generation of various first trimester decidual tissues. Deciduary secretory endometrium (DSE) is only predecidualized and will develop into decidua parietalis (DP) under influences of other tissue-type PA (tPA) or urokinase-type PA (uPA). tPA is filled with maternal blood, causing the oxygen level and the oxidative stress to rise and thereby stimulating placental differentiation and vascularization (Burton et al., 1999). This process is thought to be largely completed at 12 weeks gestation. The early group (n = 25) has a mean GA of 45.3 days (6 weeks 3 days) and the late group (n = 8) has a mean GA of 90.1 days (12 weeks 6 days). Maternal age and number of previous pregnancies and spontaneous abortions did not differ significantly between the two groups. Patient characteristics of the study groups are given in Table I.

### Materials and methods

#### Study group

Decidua samples were obtained from women (n = 32) with a viable intrauterine gravidity, undergoing a legal voluntary abortion. The study was approved by the Institutional Review Board, the ethics committee, of the Leiden University Medical Centre and informed consent was provided by all study subjects. Foetal cardiac activity and gestational age (GA) were confirmed by ultrasound. Women with symptoms of a missed abortion, such as vaginal bleeding, and women with underlying pathologies were excluded. Inconsistency between the ultrasound-determined GA and the known last day of menstruation also led to exclusion.

Two groups were formed based on a GA of <8–9 and of >8–9 weeks. This cut-off was chosen since after 8–9 weeks GA the intervillous space is gradually filled with maternal blood, causing the oxygen level and the oxidative stress to rise and thereby stimulating placental differentiation and vascularization (Burton et al., 1999). This process is thought to be largely completed at 12 weeks gestation. The early group (n = 25) has a mean GA of 45.3 days (6 weeks 3 days) and the late group (n = 8) has a mean GA of 90.1 days (12 weeks 6 days). Maternal age and number of previous pregnancies and spontaneous abortions did not differ significantly between the two groups. Patient characteristics of the study groups are given in Table I.

#### Tissue samples

Decidua samples were obtained from the aspired tissue (vacuum aspiration), fixed in formaldehyde overnight and embedded in paraffin. Haematoxylin Phloxin Safrane (HPS) and anti-cytokeratin staining were performed. Haematoxylin (50 g potassium aluminiumsulfate, 1 g haematox, 500 mg citric acid, 25 g chloralhydrate, 200 mg NaO3 in 1000 ml aqua dest) stains nuclei and calcium blue. Phloxin (0.25 g phloxin in 100 ml aquadest) stains erythrocytes, cytoplasm, fibrin and muscle red. Safrane (3 g safrane in 1000 ml 100% alcohol) stains calcium free bone, cartilage and collagen yellow. The HPS staining was used to differentiate between decidua and DSE, which microscopically resembles secretory endometrium. DB and DP were differentiated by the presence or absence of EVT using an anti-cytokeratin staining (see section immunohistochemistry).

DSE, DP and DB were obtained from the same curettement and therefore originate from the same depth and area of the uterine wall. Only subjects with at least two complete sets of DSE, DB and DP were included. Serial sections of the paraffin embedded tissue samples were used for all experiments and all parameters were compared between tissues within subjects.

#### RNA isolation and cDNA synthesis

RNA was extracted from paraffin embedded tissue samples (Specht et al., 2001; Plaisier et al., 2007). Several patients per sample were used and these samples contained a proportionate amount of cells and cell types. In short, 5 μm sections were mounted on RNase-free glass slides. The first and last sections were used to

### Table I. Characteristics of the study subjects

<table>
<thead>
<tr>
<th></th>
<th>Early first trimester group (n = 25)</th>
<th>Late first trimester group (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)*</td>
<td>29.4 ± 6.9</td>
<td>29.6 ± 14.4</td>
</tr>
<tr>
<td>GA (days)*</td>
<td>45.3 ± 7.8</td>
<td>90.1 ± 12.6</td>
</tr>
<tr>
<td>Number of previous pregnancies*</td>
<td>1.3 ± 0.3</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>Number of previous spontaneous abortions*</td>
<td>0.2 ± 0.1</td>
<td>0.4 ± 0.3</td>
</tr>
</tbody>
</table>

Values are mean ± SD.

*P > 0.05.
verify the presence of the tissues of interest. The other sections were deparafﬁnized and the tissues of interest, DSE, DP or DB (without villous tissue), were macro-dissected and dissolved in 190 µl lysis buffer (Tris 20 mM pH 7.4, EDTA 1 mM pH 8.0, 2% sodium dodecyl sulphate) and 10 µl proteinase K (20 mg/ml proteinase K, Life-Technologies Gibco BRL, Gaithersburg) for 18 h at 60°C. Subsequently, 400 µl Solution D (4 M guanidium isothiocyanate, 0.75 M sodium citrate, 10% sarkosyl and 2-mercapto-ethanol) was added and RNA was isolated (Chomczynski and Sacchi, 1987).

RNA quantity and quality were analysed in a spectrophotometer (Nanodrop ND-1000). Reverse transcription was performed with 1 µg total RNA, random primers and cDNA synthesis kit according to the manufacturer’s protocol and the obtained 32 µl cDNA was diluted 1:3 (Ready-to-go You-Prime ﬁrst strand beads, Amersham Biosciences, Buckinghamshire, UK).

**Real-time RT–PCR**

The mRNA levels were semi-quantiﬁed according to the Taqman real-time PCR method using validated primer and probe (FAM/TAMRA double-labelled) sets for MT1-, MT2-, MT3-, and MT5-MMP, uPA and uPAR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, primers/VIC-labelled probe) was used as an endogenous reference gene (all primer/probe sets purchased from Applied Biosystems, Foster City, CA, USA). Other genes, β-actin, β2-microglobulin and cyclophilin, were also used as reference genes and showed comparable results (data not shown).

RT–PCR reactions for target gene/GAPDH pairs were performed in 12.5 µl reactions, containing 2.5 µl cDNA solution using mastermix (RT-QP2X-03, Eurogentec, Maastricht, The Netherlands), DNase free water and the above primers and probe sets. Reactions were performed in duplicate and expressed in cycle threshold (Ct). A standard curve for each primer/probe pair was created by determining Ct values of various concentrations of total RNA (range 125–0.016 ng/µl). Then Ct values of the samples were quantiﬁed into ng/µl. The RNA levels in DSE were set at 100% to compare DSE with DP and DB and the RNA levels of DP were set at 100% to compare DP with DB. Water and negative-RT samples, obtained by the omission of the reverse transcriptase enzyme in the cDNA reaction, were used as negative controls.

**Immunohistochemistry**

Serial sections were deparafﬁnized, endogenous peroxidase was quenched with 3% H2O2/methanol and aspeciﬁc binding was reduced by incubation with 5% bovine serum albumin (BSA). Antigen retrieval in a trypsin solution was used for detection of MT2- and MT5-MMP (Plaisier et al., 2006). Heat retrieval in citrate buffer (pH 6.0) was used for detection of CD56 and CD68.

The following ﬁrst antibodies were used: broad spectrum polyclonal rabbit anti-cytokeratin (1:2000, Z0622, DAKO, Glostrup, Denmark), monoclonal mouse anti-CD56 (IgG1, 1:50, MONX 10844, clone B16, Monosan, Uden, The Netherlands), monoclonal mouse anti-CD68 (IgG1, 1:200, M0814, DAKO), monoclonal mouse anti-RT2-MMP (IgG1, 1:250, 162-22G5, Oncogene Research Products, San Diego, USA), monoclonal mouse anti-MT3-MMP (IgG1, 1:300, 117-10C6, Oncogene Research Products, San Diego, USA), polyclonal rabbit anti-MT5-MMP (1:200, M6684, Sigma-Aldrich, USA), monoclonal mouse anti-uPA (IgG1, 1:50, 3689, American Diagnostica Inc., Greenwich, USA). Polyclonal rabbit anti-MT1-MMP (1:1000) and anti-uPAR (1:400) 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). The following secondary antibodies were used: biotinylated horse anti-mouse antibody (1:300, BA-2000, Vector, Burlingame, USA), biotinylated donkey anti-rabbit antibody (1:300, RPN1004, Amersham Biosciences).

Primary antibodies were applied overnight at 4°C followed by 1 h incubation with biotinylated secondary antibody. Antibody binding was visualized using StreptABComplex/horse-radish peroxidase, a streptavidin complexed with biotinylated peroxidase (K0377, DakoCytomation, Glostrup, Denmark) and Novared™ substrate (SK-4800, Vector) according to the manufacturer’s protocol. Only CD56 immunohistochemistry was stained with DAB (3,3-diaminobenzidin). All incubations were performed in 1% BSA in PBS.

Sections were counterstained with Mayer’s haematoxylin. Speciﬁcity of the immunohistochemical reaction was veriﬁed by the omission of the ﬁrst antibody as well as using non-immune mouse IgG1, concentration adjusted to primary IgG1 concentration (range 0.3–20 µg/ml), and rabbit serum instead of ﬁrst antibody. To evaluate the staining patterns in EVT, cytokeratin and target protein staining were performed on serial sections of 3 µm.

**Evaluation of immunohistochemical staining**

Immunostaining of CD56+ and CD68+ cells was evaluated by counting, the number of positive cells and the number of SC in a 16 µm2 grid (10 ﬁelds per tissue per patient). In this way, the mean percentage of positive cells per SC and per µm2 could be determined.

To limit the possible bias of subjectivity during the evaluation of the immunostainings, we have chosen to evaluate the immunostaining of the proteases and uPAR by calculating a staining index (SI): proportion of stained cells × staining intensity (Nap et al., 2004; Plaisier et al., 2006, 2007). The proportion of stained cells was expressed as 0, 1, 2 or 3, which marks positive staining signal in 0%, <10%, 10–50% or >50% of the cells of a particular cell type. The intensity of staining was expressed as 1, 2 or 3 (weak, moderate or strong, respectively). The minimum score was 0 and the maximum score 9. The average score of two independent observers was used to calculate the mean and total SI (Mean and Total SI).

The Mean SI represents the protein level per studied cell type. The Total SI represents the total protein staining per tissue and was calculated as the sum of the mean SI of EC, peri-vascular smooth muscle cells (PSCM), glandular epithelium (GE) and SC in DSE and DP samples. The mean SI of EVT was also included in the total SI of DB samples.

**Statistical analysis**

All parameters were compared between DSE and DP, between DSE and DB and between DP and DB within subjects. A general linear model for repeated measurements, repeated measures ANOVA, was performed to analyse the double paired data within subjects of early ﬁrst trimester decidua as well as to compare data between the early and late ﬁrst trimester group (SPSS 11.5). Sphericity was corrected using the Greenhouse-Geisser correction. Where appropriate we used Friedman’s test for non-parametric investigations of correlated observations. The statistical analyses used are described in legends and table footnotes. P-values of <0.05 were considered signiﬁcant.

**Results**

**mRNA levels of proteases in early ﬁrst trimester decidua**

The mRNA levels of uPA, uPAR and the transmembrane spanning MT-MMPs were evaluated at the mRNA level. All these genes were expressed by the three decidual tissues (Table II).

First, the amounts of speciﬁc mRNAs in decidual parietalis (DP) were compared to those in DSE (100%). mRNA levels of uPAR and all proteases, except MT3-MMP, were elevated in DP, but only MT1-MMP (171%, P < 0.05) and uPAR (185%, P < 0.01) were signiﬁcantly increased. The mRNA levels of MT3-MMP were comparable in DP and DSE (Table II).

Subsequently, the mRNA levels in DB were compared to those DSE (100%). This comparison showed a reduction of MT5-MMP (84%, P < 0.01) and an increase of uPAR (208%, P < 0.01), MT1-MMP (198%, P < 0.05), MT2-MMP (2329%, P < 0.01) and MT3-MMP (1023%, P < 0.01) when DB compared with DSE. uPA RNA levels did not differ signiﬁcantly (Table II).

Finally, mRNA content in DB was compared with that in DP (100%). This comparison showed a reduced amount of MT5-MMP (75%, P < 0.05) and uPA (38%, P < 0.05) and markedly increased amount of MT2-MMP (1929%, P < 0.01) and MT3-MMP (768%, P < 0.01) when DB compared with DP. The concentrations of MT1-MMP and uPAR mRNA did not differ when DB compared with DP (Table II).
mRNA levels were determined by RT–PCR in DSE, DP and DB of early first trimester and values were corrected for RNA input by calculating the ratio \( \frac{\text{ng/uPA}}{\text{uPAR}} \). The mRNA expression of DP was set at 100% to compare DP with DB. Fold induction was calculated as the ratio (DB)/(DP).

### Immunohistochemistry of proteases in early first trimester decidua

The presence and cellular localization of the six proteins involved in pericellular proteolysis were determined at the protein level in serial sections of DSE, DP and DB. The studied proteins were detectable in all decidual tissues, and their presence was graded as mean staining indices (mean SI) per cell type and total staining indices (total SI) based on the sum of the cell types-dependent indices (Table III and Figs. 1–3).

The total staining indices in DSE were comparable to those in DP with only a higher total SI for MT1-MMP in DP (Table III, \( P < 0.05 \)). Secondly, when DSE and DB were compared, only an elevated total SI for uPA was found when DB compared with DSE (Table III, \( P < 0.05 \)). Thirdly, DB was compared to DP and this showed a similar pattern; only an elevated total SI for uPA when DB compared with DP (Table III, \( P < 0.05 \)).

### Table II. Differential mRNA levels in early first trimester pregnancies (percentage ± SD)

<table>
<thead>
<tr>
<th></th>
<th>DSE(^a)</th>
<th>DP</th>
<th>DB</th>
<th>DP (b)</th>
<th>DB</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT1-MMP</td>
<td>100 ± 14%</td>
<td>171 ± 29%*</td>
<td>198 ± 52%*</td>
<td>100%</td>
<td>155 ± 44%</td>
</tr>
<tr>
<td>MT2-MMP</td>
<td>100 ± 33%</td>
<td>308 ± 119%</td>
<td>2320 ± 428%**</td>
<td>100%</td>
<td>1929 ± 316%**</td>
</tr>
<tr>
<td>MT3-MMP</td>
<td>100 ± 30%</td>
<td>150 ± 32%</td>
<td>1023 ± 288%**</td>
<td>100%</td>
<td>768 ± 119%**</td>
</tr>
<tr>
<td>MT5-MMP</td>
<td>100 ± 12%</td>
<td>70 ± 8%</td>
<td>16 ± 2%**</td>
<td>100%</td>
<td>25 ± 3%*</td>
</tr>
<tr>
<td>uPA</td>
<td>100 ± 20%</td>
<td>232 ± 74%</td>
<td>122 ± 31%</td>
<td>100%</td>
<td>62 ± 16%*</td>
</tr>
<tr>
<td>uPAR</td>
<td>100 ± 13%</td>
<td>185 ± 22%**</td>
<td>208 ± 17%**</td>
<td>100%</td>
<td>128 ± 19%</td>
</tr>
</tbody>
</table>

mRNA levels were determined by RT–PCR in DSE, DP and DB of early first trimester \( n = 25 \) and compared within subjects. Mean Ct per gene: GAPDH \( 25–26 \), MT1-MMP \( 27 \), MT2-MMP \( 30 \), MT3-MMP \( 30 \), MT5-MMP \( 29 \), uPA \( 31 \), uPAR \( 30 \). Ct were converted into ng/uPA using a standard curve of total RNA and the 'repeated measures ANOVA' was performed to analyse the data.

The mRNA levels of DSE was set at 100% to compare mRNA levels in DSE with those in DP and DB. Fold induction was calculated as the ratio (DP)/(DSE) and (DB)/(DSE). The mRNA expression of DP was set at 100% to compare DP with DB. Fold induction was calculated as the ratio (DB)/(DP).

\( *P < 0.05; **P < 0.01 \)

### Table III. First trimester protein levels in early versus late first trimester pregnancies

<table>
<thead>
<tr>
<th></th>
<th>EC(^a)</th>
<th>PSMC(^a)</th>
<th>GE(^a)</th>
<th>SC(^a)</th>
<th>EVT(^a)</th>
<th>Total SI(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early first</td>
<td>Late first</td>
<td>Early first</td>
<td>Late first</td>
<td>Early first</td>
<td>Late first</td>
</tr>
<tr>
<td>MT1</td>
<td>DSE 0.6</td>
<td>0.0**</td>
<td>1.8</td>
<td>0.3</td>
<td>4.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>DP 0.7</td>
<td>0.0**</td>
<td>2.2</td>
<td>0.3</td>
<td>4.8</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>DB 0.6</td>
<td>0.0*</td>
<td>1.7</td>
<td>1.0</td>
<td>3.7</td>
<td>3.0</td>
</tr>
<tr>
<td>MT2</td>
<td>DSE 1.3</td>
<td>0.5</td>
<td>3.4</td>
<td>0.7</td>
<td>4.2</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>DP 1.0</td>
<td>0.3*</td>
<td>3.7</td>
<td>1.5</td>
<td>4.3</td>
<td>2.7</td>
</tr>
<tr>
<td>MT3</td>
<td>DSE 0.5</td>
<td>0.0</td>
<td>2.2</td>
<td>0.7</td>
<td>4.8</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>DP 0.1</td>
<td>0.0</td>
<td>2.4</td>
<td>0.7</td>
<td>4.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>DB 0.0</td>
<td>0.0</td>
<td>1.6</td>
<td>1.0</td>
<td>5.2</td>
<td>1.0</td>
</tr>
<tr>
<td>MT5</td>
<td>DSE 0.0</td>
<td>0.0</td>
<td>0.3</td>
<td>0.0</td>
<td>1.7</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>DP 0.0</td>
<td>0.0</td>
<td>0.3</td>
<td>0.0</td>
<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>DB 0.0</td>
<td>0.0</td>
<td>0.2</td>
<td>0.0</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>uPA</td>
<td>DSE 0.2</td>
<td>0.1</td>
<td>1.8</td>
<td>2.0</td>
<td>4.4</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>DP 0.3</td>
<td>0.3</td>
<td>1.8</td>
<td>1.7</td>
<td>4.4</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>DB 0.4</td>
<td>0.5</td>
<td>2.1</td>
<td>2.0</td>
<td>5.4</td>
<td>5.3</td>
</tr>
<tr>
<td>uPAR</td>
<td>DSE 0.3</td>
<td>0.5</td>
<td>0.9</td>
<td>1.8</td>
<td>5.6</td>
<td>3.8</td>
</tr>
<tr>
<td></td>
<td>DP 0.2</td>
<td>0.0</td>
<td>0.7</td>
<td>1.7</td>
<td>5.5</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>DB 0.4</td>
<td>0.8</td>
<td>0.7</td>
<td>2.2</td>
<td>5.4</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Specific antigens of pericellular acting proteases in DSE, DP and DB were determined by immunohistochemistry, scored per cell type and compared within subjects. Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis.

NP, not present.

\( a \) The staining was expressed as mean staining indices per cell type and as the total staining indices (total SI), which is the sum of the mean staining indices of EC, PSMC, GE, SC and EVT per decidual tissue.

\( b \) All proteases studied were present in EVT, although MT5-MMP, MT5-MMP was not detectable (Fig. 2). Significant differences were not found between the three decidual tissues with regard to endothelial protein levels. However, it should be noted that MT3-MMP was present in the endothelium of DSE, but largely reduced in DP and undetectable in those cells of DB. The same pattern was also displayed by endothelial MT2-MMP and by the staining of MT2- and MT3-MMP in smooth muscle cells (Table III and Figs. 1 and 2).

\( aP < 0.05 \) in DSE versus DP; \( bP < 0.05 \) in DB versus DP and DSE in the early first trimester group; \( *P < 0.05 \) in late versus early first trimester decidua.

Analysis of the protein staining in the various cell types showed that the mean staining indices were most abundant in glandular epithelial cells, followed by PSMC and SC (Table III and Fig. 1). Endothelium displayed weak staining for all proteins, except MT5-MMP. MT5-MMP was not detectable (Fig. 2). Significant differences were not found between the three decidual tissues with regard to endothelial protein levels. However, it should be noted that MT3-MMP was present in the endothelium of DSE, but largely reduced in DP and undetectable in those cells of DB. The same pattern was also displayed by endothelial MT2-MMP and by the staining of MT2- and MT3-MMP in smooth muscle cells (Table III and Figs. 1 and 2).
whereas it was abundantly present in syncytiotrophoblasts (Table III and Fig. 3).

Proteases in early versus late first trimester decidua

The mRNA levels of proteases in late first trimester decidua were compared with those in early first trimester decidua (100%, Table IV). The levels of MT1-MMP (206%, \( P < 0.02 \)) and MT2-MMP (1047%, \( P < 0.02 \)) mRNAs were elevated, whereas MT3-MMP mRNA content was reduced (62%, \( P < 0.05 \)) in DSE of late compared with early first trimester pregnancies. Comparison of DP between both groups showed elevated mRNA levels of MT1-MMP (215%, \( P < 0.02 \)) and uPAR (207%, \( P < 0.02 \)) and reduced mRNA levels of MT3-MMP (77%, \( P < 0.05 \)) in late first trimester tissues. The mRNA levels of these proteases in DB showed no differences between the two groups (Table IV).

At the protein level, total and mean staining indices of MT1- and MT2-MMP were reduced in DSE and DP of late first trimester compared to early first trimester (\( P < 0.05 \)). The total and mean SI of MT3-MMP were reduced in all cell types of all tissues in late first trimester decidua (Table III, \( P < 0.05 \)).

The mean SI of MT1-, MT2- and MT3-MMP in endothelium were reduced in late versus early first trimester DSE, DP and DB. Strikingly, endothelial MT1 and MT3-MMP mRNAs were not even detectable in late first trimester decidua. uPA and uPAR displayed comparable staining indices in EC at the two time points, whereas MT5-MMP was absent in these cells in both conditions (Table III).

Analysis of uNK cells (CD56+) and macrophages (CD68+)

The presence of CD56 and CD68 antigens was determined in DSE, DP and DB of both the early and late first trimester group and expressed as the percentage positive cells per total number of SC.

The CD56 staining showed inhomogeneous staining, mainly localized around vessels and glands, which was corrected for by analysing various samples per patient. The number of CD56+ (uNK) cells in DP and DB was comparable, 18 ± 6% and 21 ± 4%, respectively, of the total number of SC. Both DP and DB showed a significantly larger number of CD56+ cells than DSE (9 ± 2%, \( P < 0.05 \)). No significant differences were detected between early and late first trimester decidua (Fig. 4A and C).

The CD68 staining showed scattered positive cells throughout the tissues (range 1.0–2.2%). Although the number of macrophages appeared to increase from DSE to DP to DB, these differences were not significant. The two time points also showed no significant differences (Fig. 4B and D).

Discussion

The present study demonstrates that the mRNA and protein content of the pericellular-acting MT-MMPs and uPA/uPAR varied between DSE, DB and DP. Furthermore, the percentage of CD56-positive cells was two-fold lower in DSE than in DP. A comparable increase in macrophages did not reach statistical significance. It is likely that the differential expression of these proteases is mainly due to the different effects of pregnancy-induced hormones (hCG, E2 and progesterone), the EVT and/or immune cells on the three decidual tissues. Our study suggests that uPAR and MT1-MMP are enhanced by pregnancy-induced hormones, and possibly uNK cells, and uPA, MT2-, MT3-MMPs by EVT. In addition, MT5-MMP appears reduced by the EVT. Finally, we show differences in protease content and presence of immune cells as gestation progresses.

Decidual tissues and their contributors

Endometrial adaptation to pregnancy is induced by pregnancy-induced hormones, i.e. hCG, E2 and progesterone.
immune cells and the EVT. Pregnancy-induced hormones are involved in the development of DP from the DSE, whereas the additional presence of the EVT induces the generation of the DB. This may imply that differences in protease content between DB and DP are due to interactions of the decidual tissue with the trophoblast. However, NK cells have also been suggested to vary between term DB and DP (Sindram-Trujillo et al., 2003). As these uNK cells are known to produce many chemokines and angiogenic growth factors (Li et al., 2001; Hanna et al., 2006; Lash et al., 2006), the uNK cells may also be able to influence the production of proteases in DB and DP. Other investigators were unable to detect differences in uNK numbers in DP and DB (Khong, 1987; Haller et al., 1995; Bulmer and Lash, 2005). Indeed, in our study we also found similar amounts of uNK cells in DB and DP being 18 and 21%, respectively. This excludes an involvement of the number of uNK on the difference between DB and DP, but possible differences in the functional properties of the uNK cells in DB and DP cannot yet be excluded.

The percentage contribution of uNK cells and macrophages to the immune cell and overall SC populations were comparable with those reported by other investigators (Bulmer et al., 1991; Quenby et al., 1999; Tuckerman et al., 2007). Interestingly, the amounts of uNK cells were two-fold higher in DB and DP than in DSE (9%). A comparable increase in macrophages did not reach statistical significance (range 1.0–2.2% of total SC). To our knowledge, the difference between DSE and DB/DP has not been systematically investigated before.

The foregoing information is summarized in Fig. 5, which presents a model that describes the influences of uNK cells, EVT and pregnancy-induced hormones on DSE, DP and DB. This model enables us to discuss changes that occur independently of trophoblast invasion, and those that are mainly induced by pregnancy-induced hormones and/or uNK cells, from changes influenced by the invasive EVT (Plaisier et al., 2007). The effects of uNK cells require further functional studies.

**Pericellular proteases in early first trimester decidua**

MT1-MMP mRNA and protein expression were significantly induced in DB and DP when compared with DSE, but no differences were
found between DB and DP. This suggests that decidual MT1-MMP expression is induced rather by pregnancy-induced hormones and/or uNK cells then by invasive trophoblasts (EVT). This confirms an earlier study which showed that invasive trophoblasts are not required for the induction of MT1-MMP (Nakano et al., 2001). Furthermore, the apparent induction of MT1-MMP in DP and DB might be explained by the influx of NK cells in those tissues, as NK cells are known to contain MT1-MMP (Albertsson et al., 2000). MT1-MMP may also play a role in uNK cell migration.

Although the regulation of MT1-MMP expression seems unaffected by the EVT, MT1-MMP is expressed by the EVT. This suggests a role in trophoblast invasion, which indeed has been reported previously (Nawrocki et al., 1996; Hurskainen et al., 1998; Tanaka et al., 1998). Finally, the fact that MT1-MMP is induced in various cell types of DP and DB also points to a role in decidual remodelling and/or vascularization.

MT2-MMP and MT3-MMP mRNA levels were increased and MT5-MMP mRNA levels were reduced in DB compared to both DSE and DP. This suggests that these mRNA levels are mainly influenced by the presence of the EVT. Together with their antigens being present in invasive trophoblasts, this suggests a role in trophoblast invasion. However, no significant differences were detected in the overall presence of MT2-, MT3- and MT5-MMP proteins between the three types of decidua. MT2-MMP and MT3-MMP protein levels were reduced in endothelium and PSMC of DP and DB as compared to DSE. Endothelial protein expression of the other proteases showed no differences between the tissues. Interestingly, we recently determined the vascularization pattern in serial sections of the same specimens, which showed highly enhanced vascularization at the implantation site. Comparison of these data with the endothelial expression of MT2- and MT3-MMPs showed no differences between the tissues. Interestingly, we recently determined the vascularization pattern in serial sections of the same specimens, which showed highly enhanced vascularization at the implantation site. Comparison of these data with the endothelial expression of MT2- and MT3-MMPs showed no differences between the tissues. Interestingly, we recently determined the vascularization pattern in serial sections of the same specimens, which showed highly enhanced vascularization at the implantation site.

Table IV. mRNA levels in late first trimester pregnancies compared with early first trimester pregnancies (100%)

<table>
<thead>
<tr>
<th></th>
<th>Late first DSEa</th>
<th>Late first DPa</th>
<th>Late first DBa</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT1-MMP</td>
<td>206%**</td>
<td>215%**</td>
<td>150%</td>
</tr>
<tr>
<td>MT2-MMP</td>
<td>1047%**</td>
<td>80%</td>
<td>110%</td>
</tr>
<tr>
<td>MT3-MMP</td>
<td>38%*</td>
<td>23%*</td>
<td>92%</td>
</tr>
<tr>
<td>MT5-MMP</td>
<td>111%</td>
<td>98%</td>
<td>107%</td>
</tr>
<tr>
<td>uPA</td>
<td>131%</td>
<td>205%</td>
<td>168%</td>
</tr>
<tr>
<td>uPAR</td>
<td>160%</td>
<td>207%**</td>
<td>137%</td>
</tr>
</tbody>
</table>

mRNA levels was determined by RT–PCR in DSE, DP and DB of early and late first trimester (n = 25 and n = 8, respectively) and compared within subjects. The ‘repeated measures ANOVA’ was performed to analyse the data.

aThe mRNA levels in DSE, DP and DB of early first trimester decidua were set at 100% to compare with mRNA levels in DSE, DP and DB of late first trimester decidua.

*p < 0.05; **p < 0.02.

Figure 3: Protein levels of proteases in villous and EVT of early first trimester decidua

The presence of protease antigens in villous and EVT in DB was studied in serial sections stained against cytokeratin and the target protein. (A) Immunostaining in syncyto- and cytotrophoblasts (synCTB and CTB) was expressed as the mean SI ± SEM. (B) uPAR (closed arrows) and epithelium (open arrows) in serial sections. (D) uPA antigen in syncytiotrophoblast (open arrow) and not in cytotrophoblast (closed arrow). (B–D) Bar = 50 μm.

Table IV. mRNA levels in late first trimester pregnancies compared with early first trimester pregnancies (100%)

<table>
<thead>
<tr>
<th></th>
<th>Late first DSEa</th>
<th>Late first DPa</th>
<th>Late first DBa</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT1-MMP</td>
<td>206%**</td>
<td>215%**</td>
<td>150%</td>
</tr>
<tr>
<td>MT2-MMP</td>
<td>1047%**</td>
<td>80%</td>
<td>110%</td>
</tr>
<tr>
<td>MT3-MMP</td>
<td>38%*</td>
<td>23%*</td>
<td>92%</td>
</tr>
<tr>
<td>MT5-MMP</td>
<td>111%</td>
<td>98%</td>
<td>107%</td>
</tr>
<tr>
<td>uPA</td>
<td>131%</td>
<td>205%</td>
<td>168%</td>
</tr>
<tr>
<td>uPAR</td>
<td>160%</td>
<td>207%**</td>
<td>137%</td>
</tr>
</tbody>
</table>

mRNA levels was determined by RT–PCR in DSE, DP and DB of early and late first trimester (n = 25 and n = 8, respectively) and compared within subjects. The ‘repeated measures ANOVA’ was performed to analyse the data.

aThe mRNA levels in DSE, DP and DB of early first trimester decidua were set at 100% to compare with mRNA levels in DSE, DP and DB of late first trimester decidua.

*p < 0.05; **p < 0.02.

Proteases in human first trimester decidua

47
and SC contained uPA antigen and GE did not (Koolwijk et al., 2001), glandular epithelial cells are positive for uPA antigen in first trimester tissues together with the endothelium and part of the SC. The expression of uPAR largely follows the pattern of uPA and is clearly present in EC. The presence of uPAR in EVT is confirmed by previous studies and suggests a role in trophoblast invasion (Pierleoni et al., 1998; Floridon et al., 1999).

Interestingly, the expression of uPA mRNA and antigen showed different patterns. The mRNA levels suggest a stimulatory influence of pregnancy-induced hormones and/or uNK cells, as well as an inhibiting influence of the EVT. This latter observation may be explained by the down-regulation of uPA mRNA by hCG, an important product of trophoblasts (Yagel et al., 1993; Salamonsen 1999). However, uPA was increased at the protein level in all cell types when DB compared with DP and DSE. The presence of uPA is not only determined by the ability of the cells to produce uPA, but also by their content of uPAR, which binds and internalizes uPA in complex with its inhibitor PAI-1 (Kroon et al., 1989; Blasi and Carmeliet, 2002). Furthermore, uPA and uPAR are present in a part of the SC that probably include the uNK and other leukocytes. This is in concert with the notion that the uPA/uPAR system is used, probably together with MT-MMPs, by leukocytes for their invasion into tissues (Albertsson et al., 2000; Al-Atrash et al., 2001). Moreover, the increase in uNK cells may thus contribute to the increased uPA antigens observed in DB as compared to DSE.

**Early versus late first trimester pregnancies**

The expression of uPA and uPAR mRNA and proteins showed no differences between early and late trimester decidua, indicating that the uPA/uPAR system remains under a constant regulation as gestation progresses. No data are available in literature regarding uPA/uPAR during these time points. In contrast, the differential mRNA expression of the MMPs suggests that these proteases are regulated over time. The overall amounts of MT1-, MT2- and MT3-MMP were reduced in all decidual tissues in late compared with early first trimester pregnancy.

Furthermore, the MT1- MT2- and MT3-MMP antigen expression in endothelium and PSMC was also reduced in all types of decidua of late first trimester pregnancy. We recently determined the vascularization pattern in serial sections of the same early and late first trimester decidua samples and this showed that vascularization is regulated as gestation progresses. The endothelial expression of MT1-, MT2- and MT3-MMPs correlated well with the differences in vascularization between early and late first trimester decidua (Plaisier et al., 2007). These data again point to a role for these MT-MMPs in determining the degree and pattern of neovascularization as gestation progresses.

**Conclusion**

Decidual adaptation is important in the development of a healthy pregnancy. We showed that the expression of various pericellular-acting
Proteases varied between DSE, DB and DP. Furthermore, uNK cells (CD56+ and macrophages (CD68+) were present in all decidual tissues and only the percentage of uNK cells differed between the tissues. The differential presence of several proteases enabled hypothesizing about their regulation, i.e. by pregnancy-induced hormones, the EVT and/or immune cells, as well as their functions, e.g. in immune cell infiltration, trophoblast invasion and/or vascularization (Fig. 5).

uPAR and MT1-MMP expression appeared regulated by pregnancy-induced hormones and/or uNK cells, whereas the presence of uPA, MT2-, MT3- and MT5-MMP appeared regulated by the EVT. All proteases were expressed by the EVT and might be involved in trophoblast invasion. MT2- and MT3-MMP are known candidates in regulating angiogenesis and together with their differential expression this suggests that they may not only support decidual remodelling and trophoblast invasion but also, partially, account for the vascular changes at the implantation site. Finally, we show differences in protease expression as gestation progresses. A better understanding of decidualization may contribute to new insights in currently non-explicable pathological events associated with pregnancy, such as spontaneous abortions and pre-eclampsia.

Acknowledgements
We would like to thank M. de Vries and Prof. Dr J.W.M. Niessen for their help regarding immune cell immunohistochemistry.

References
Plaisier et al.


Submitted on August 24, 2007; resubmitted on November 4, 2007; accepted on November 30, 2007.