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

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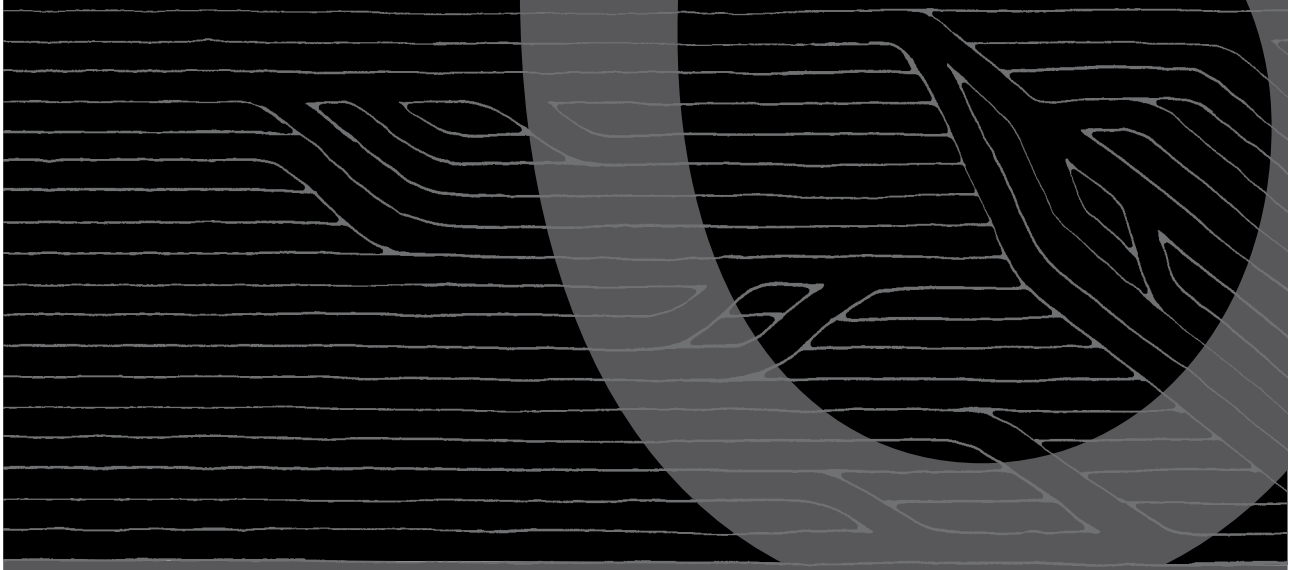
Chapter 6

Pericellular-acting proteases in human first-trimester decidua.



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ABSTRACT

Objective: 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 and its receptor uPAR in decidua. Therefore, their protein and mRNA expressions were analysed in three first-trimester decidual tissues, decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB).

Methods: 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.

Results/conclusion: 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 prominent 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 peri-cellular proteases in decidua by pregnancy-induced hormones, immune cells and EVT.

INTRODUCTION

Proteolysis is essential for decidual remodelling and vascularisation during implantation. Disturbances in decidual development may play a role in the pathogenesis of miscarriages and pre-eclampsia [Solberg *et al.,* 2003; Vailhe *et al.,* 1999; Vuorela *et al.,* 2000; Zygmunt *et al.,* 2003]. Endometrial adaptation to implantation starts during the receptive secretory phase of the menstrual cycle and continues throughout the first-trimester. This adaptation includes decidualisation, tissue remodelling, angiogenesis, and immune cell invasion. The invasion of immune cells is enormous: from 8% of total stromal cells during the menstrual cycle up to 30% during the first-trimester. Approximately 70% of these leucocytes are uNK cells and 10% are macrophages [Bulmer *et al.,* 1991].
Decidual remodelling, cell invasion as well as angiogenesis are facilitated by proteolysis. Trophoblasts, uNK cells and endothelial cells require proteolytic activity to degrade their extra cellular matrix (ECM) proteins and to migrate [Al Atrash *et al.,* 2001; Heymans *et al.,* 1999; Pepper 2001; Stetler-Stevenson 1999; Salamonsen 1999]. These cells are able to generate proteolytic activity by either producing proteases or using proteases on neighbouring cells and/or ECM [Albertsson *et al.,* 2000; Kim *et al.,* 2000; Koolwijk *et al.,* 2001; Salamonsen 1999; Van den Heuvel *et al.,* 2005b]. Key regulators of proteolysis belong to the family of matrix metalloproteinases (MMPs), in particular to the subgroup of membrane-type matrix metalloproteinases (MT-MMPs), and to the plasmin/plasminogen system [Alfano *et al.,* 2005; Kindzelskii *et al.,* 2004; Reuning *et al.,* 2003].

The membrane-associated localisation of MT-MMPs makes them suited for pericellular proteolysis [Egeblad, Werb 2002; Hotary *et al.,* 2000; Seiki, Yana 2003; Van Hinsbergh *et al.,* 2006]. We studied the transmembrane-spanning MT-MMPs, MT1- (MMP-14), MT2- (MMP-15), MT3- (MMP-16) and MT5-MMP (MMP-24). Only these MT-MMPs have the proteolytic potential to induce capillary tube formation, while the GPI-anchored MT4- and MT6-MMP were unable to do so [Hotary *et al.,* 2000]. 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, 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 [Collen *et al.,* 2003; Galvez *et al.,* 2001; Hiroaka *et al.,* 1998; Lafleur *et al.,* 2002; Seiki, Yana 2003; Sounni *et al.,* 2002; Visse, 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* [Collen *et al.,* 2003; Galvez *et al.,* 2001; Hiroaka *et al.,* 1998; Hotary *et al.,* 2000; Hotary *et al.,* 2002; Lafleur *et al.,* 2002; 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, stromal cells, and the extra-villous trophoblast [Bai *et al.,* 2005; Bjorn *et al.,* 2000; Curry, Osteen 2003; Hurskainen *et al.,* 1998; Nakano *et al.,* 2001; Nawrocki *et al.,* 1996]. MT-MMPs are assumed to regulate trophoblast invasion during implantation [Salamonsen 1999]. Whether migration of other cell types, e.g. immune and endothelial cells, 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 plasminogen activator (tPA) or urokinase-type plasminogen activator (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, plasminogen activator inhibitor type-1 and -2 (PAI-1/2) [Spengers, Kluft 1987].
The role of uPA mediated plasminogen activation in cell migration has been studied for a variety of cells and for endothelial cells, leucocytes, and trophoblasts in particular [Heymans *et al.,* 1999; Reuning *et al.,* 2003; Pepper 2001; Blasi *et al.,* 1987; Hu *et al.,* 1999; 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 play 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, vascularisation, and immune cell and trophoblast invasion is not well established.
Endometrial adaptation to pregnancy is induced by pregnancy-induced hormones, i.e. hCG, oestradiol and progesterone, immune cells, and the extra-villous trophoblasts (EVT). The differential presence of these factors results in the generation of various first-trimester decidual tissues. Decidual secretory endometrium (DSE) is only pre-decidualised and will develop into decidua parietalis (DP) under influence of pregnancy-induced hormones. Decidua basalis (DB) will arise in the additional presence of the EVT. The present study analyses the expression of MT-MMPs and uPA/uPAR at mRNA and pro-

tein level in decidual secretory endometrium (DSE), decidua parietalis (DP) and decidua basalis (DB). These parameters will be compared between tissues within subjects. In this way, MT-MMP and uPA/uPAR expression in various decidual cell types and decidual tissues can be determined. As the influx and activity of immune cells, in particular uNK cells and macrophages, may influence that production of proteases, we also compared the accumulation of these cells in the same decidual tissues. These data may shed light on the functions of the studied proteases in human decidua and might generate new hypotheses regarding the inception of pregnancy.

MATERIALS AND METHODS

Study group

Decidua samples were obtained from women (n=32) with a viable intrauterine gravidity, undergoing a legal voluntarily 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 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 gestational age and the known last day of menstruation also led to exclusion.

Two groups were formed based on a gestational age (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 vascularisation [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^{+3} weeks) and the late group (n=8) has a mean GA of 90.1 days (12^{+6} weeks). Maternal age and number of previous pregnancies and miscarriages did not differ significantly between the two groups. Patient characteristics of the study groups are given in Table 1.

Tissue Samples

Decidua samples were obtained from the aspirated tissue (vacuum aspiration), fixed in formaldehyde overnight and embedded in paraffin. Hematoxylin Phloxin Safrane (HPS) and anti-cytokeratin staining were performed. Hematoxylin (50 g potassium aluminium-sulfate, 1 g haematox, 500 mg citric acid, 25 g chloralhydrate, 200 mg $NaJO_3$ in 1000 ml aqua dest,) stains nuclei and calcium blue. Phloxin (0.25 g phloxin in 100 ml aquadest) stains erytrocytes, 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

Table 1. Characteristics of the study subjects.

	Early first- trimester group (*n*=25) Mean ± SD	**Late first- trimester group** (*n*=8) Mean ± SD
Maternal Age (years)[a]	29.4 ± 6.9	29.6 ± 14.4
Gestational age (days)[a]	45.3 ± 7.8	90.1 ± 12.6
Number of previous pregnancies[a]	1.3 ± 0.3	1.6 ± 0.5
Number of previous miscarriages[a]	0.2 ± 0.1	0.4 ± 0.3

[a] p>0.05.

was used to differentiate between decidua and decidual secretory endometrium (DSE), which microscopically resembles secretory endometrium. Decidua basalis (DB) and decidua parietalis (DP) were differentiated by the presence or absence of extra-villous trophoblasts using an anti-cytokeratin staining (see section immunohistochemistry, Figure 5).

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 set 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 [Plaisier *et al.,* 2007; Specht *et al.,* 2001]. Several samples per patient 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 verify the presence of the tissues of interest. The other sections were deparaffinised 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% SDS) and 10 μl proteinase K (20 mg/ml proteinase K, Life-Technologies Gibco BRL, Gaithersburg) for 18 hours 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, 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. glycerlylaldehyde'-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, β_2-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 described primer/probe sets. Reactions were performed in duplicate, 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. RNA levels in DSE was set at 100% to compare DSE with DP and DB and the gene expression of DP was 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 deparaffinised, 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 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 (IgG_1, 1:50, MONX 10844, clone 1B6, Monosan, Uden, the Netherlands), monoclonal mouse anti-CD68 (IgG_1,1:200,M0814, DAKO,GlostrupDenmark), monoclonal mouse anti-MT2-MMP (IgG_1, 1:250, 162-22G5, Oncogene Research Products, San Diego, USA), monoclonal mouse anti-MT3-MMP (IgG_1, 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 (IgG_1, 1:50, 3689, American Diagnostica Inc, Greenwich, USA). Polyclonal rabbit anti-MT1-MMP (1:1000) and anti-uPAR (1:400) antibodies were produced and characterised as previously described [Collen *et al.,* 2003; Plaisier *et al.,* 2004; van Boheemen *et al.,* 1995; Koolwijk *et al.,* 1996]. 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, Buckinghamshire, UK).

Primary antibodies were applied overnight at 4°C followed by one hour incubation with biotinylated secondary antibody. Antibody binding was visualised using StreptABComplex/HRP, a streptavidin complexed with biotinylated peroxidase (K0377, DakoCytomation, Glostrup, Denmark) and NovaRED™ substrate (SK-4800, Vector, Burlingame, USA) according to the manufacturer's protocol. Only CD56 immunohistochemistry was stained with DAB (diaminobenzidin). All incubations were performed in 1% bovine serum albumin 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 IgG_1, concentration adjusted to primary IgG_1 concentration, and rabbit serum instead of first antibody. To evaluate the expression in extra-villous trophoblast, 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 stromal cells in a 16 μm^2 grid (10 fields per tissue per patient). In this way, the mean percentage of positive cells per stromal cells and per μm^2 could be determined.
To limit the possible bias of subjectivity during the evaluation of the immunostaining, we have chosen to evaluate the immunostaining of the proteases and uPAR by calculating a staining index (SI): proportion of stained cells x staining intensity [Plaisier *et al.,* 2006; Plaisier *et al.,* 2007; Nap *et al.,* 2004]. 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 staining index (Mean and Total SI).
The Mean SI represents the protein expression per studied cell type. The Total SI represents the total protein expression per tissue and was calculated as the sum of the Mean SI of endothelial cells (EC), perivascular smooth muscle cells (PSMC), glandular epithelium (GE) and stromal cells (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 used statistical analyses 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 expression 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 2).

First, the amounts of specific mRNAs in decidual parietalis (DP) were compared to those in decidua secretory endometrium (DSE, 100%). mRNA levels of uPAR and all proteases, except MT5-MMP, were elevated in DP, but only MT1-MMP (171%, p<0.05) and uPAR (185%, p<0.01) were significantly induced. The mRNA levels of MT5-MMP, were comparable in DP and DSE (Table 2).

Subsequently, the mRNA levels in decidua basalis (DB) were compared to those DSE (100%). This comparison showed a reduction of MT5-MMP (84%, p<0.01) and an induction of uPAR (208%, p<0.01), MT1-MMP (198 %, p<0.05), MT2-MMP (2329%, p<0.01)

Table 2. Differential mRNA levels in early first-trimester pregnancies (percentage ± SD) [a].

	DSE [b]	DP	DB	DP [c]	DB
MT1-MMP	100 ± 14 %	171 ± 29 %#	198 ± 52 %#	100 %	155 ± 44 %
MT2-MMP	100 ± 33 %	308 ± 119 %	2329 ± 428 %*	100 %	1929 ± 316 %*
MT3-MMP	100 ± 30 %	150 ± 32 %	1023 ± 288 %*	100 %	768 ± 119 %*
MT5-MMP	100 ± 12 %	70 ± 8 %	16 ± 2 %*	100 %	25 ± 3 %#
uPA	100 ± 20 %	232 ± 74 %	125 ± 31 %	100 %	62 ± 16 %#
uPAR	100 ± 13 %	185 ± 22 %*	208 ± 27 %*	100 %	128 ± 19 %

[a] mRNA levels were determined by RT-PCR in DSE, DP and DB of early 1st trimester (n=25) and compared within subjects. Mean cycle threshold (Ct) per gene: GAPDH 25-26, MT1-MMP 27, MT2-MMP 30, MT3-MMP 30, MT5-MMP 29, uPA 31, uPAR 30. Cycle threshold were converted into ng/◂l using a standard curve of total RNA and values were corrected for RNA input by calculating the ratio (ng/◂l (target gene))/ (ng/◂l (GAPDH)) The 'repeated measures ANOVA' was performed to analyse the data.

[b] The mRNA expression 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).

[c] 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.

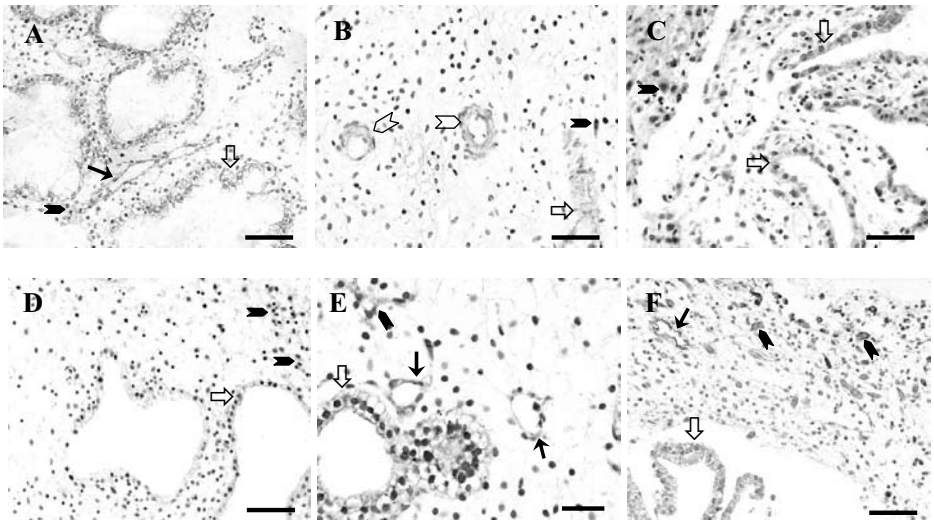

Figure 1. Protein levels of proteases in early first-trimester decidua.
The level of proteases was determined in DSE, DP and DB. **A.** MT1-MMP antigen was detectable in epithelial (open arrow), endothelium (closed arrow) and stromal cells (closed arrowhead) of DSE. **B.** MT2-MMP antigen in epithelium (open arrow), stromal cells (closed arrowhead) and pericytes (open arrowhead) of DB. **C.** Epithelial (open arrow) and stromal (closed arrowhead) MT3-MMP antigen in DB. Pericytes not present in this field. **D.** MT5-MMP antigen in epithelium (open arrow), and only dimly in stromal cells (closed arrowheads) of DP. **E.** uPA antigen in epithelium (open arrow), stromal cells (closed arrowhead) and endothelium (closed arrows) in DB. Pericytes not present in this field. **F.** Epithelial (open arrow), endothelial (closed arrows) and stromal cells (closed arrowheads) uPAR protein in DB. **A-D,F** Bar = 100 μm, **E.** Bar = 50 μm.
(see colour figures supplement)

and MT3-MMP (1023%, $p<0.01$) in DB compared to DSE. The expression of uPA did not differ significantly (Table 2).

Finally, mRNA content in DB was compared to 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 expression of MT2-MMP (1929%, $p<0.01$) and MT3-MMP (768%, $p<0.01$) in DB compared to DP. The concentrations of MT1-MMP and uPAR mRNA did not differ in DB compared to DP (Table 2).

Immunohistochemistry of proteases in early first-trimester decidua

The presence and cellular localisation 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 3, Figure 1, 2 and 3).

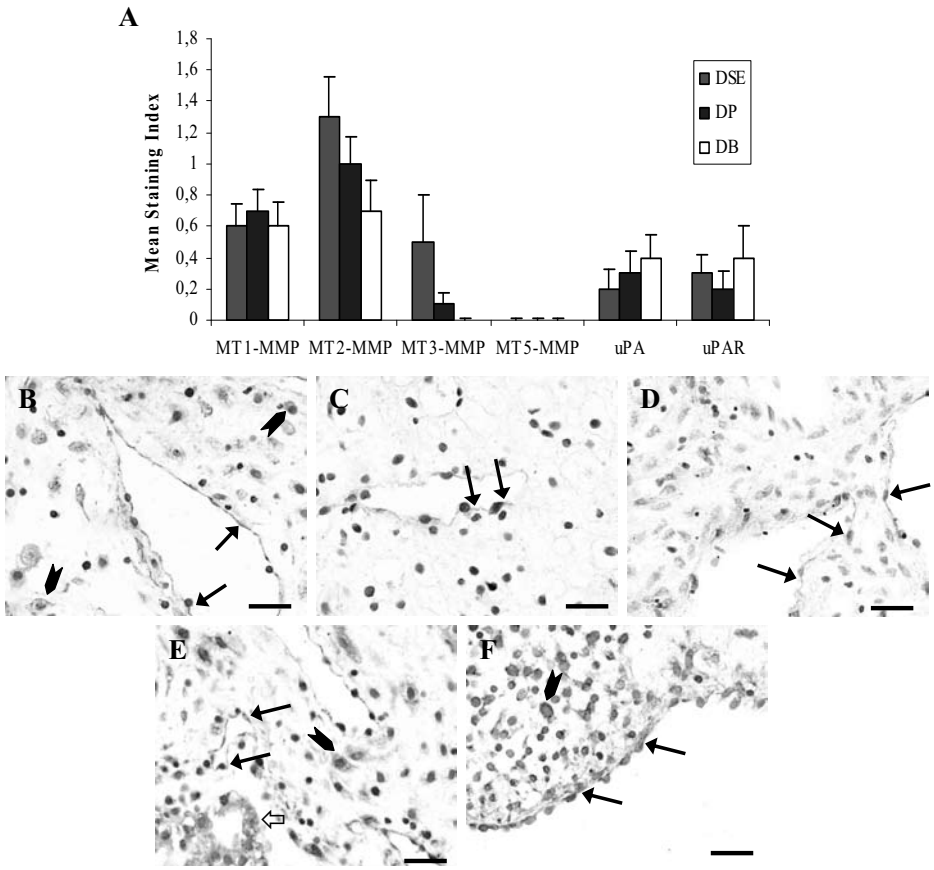

Figure 2. Protein levels of proteases in endothelium of early first-trimester decidua.
A. Presence of protease antigens in endothelium (EC) was expressed as the mean staining index ± SEM and **B-F** show examples of endothelial protease expression. **B.** Endothelial (arrows) and stromal (arrowheads) MT1-MMP was detectable in DB. **C.** MT2-MMP was detected in endothelium (arrows) of DB. **D.** Endothelial MT3-MMP antigen (arrows) in DSE. **E.** uPA antigen in endothelium (closed arrows), epithelium (open arrow) and stromal cells (arrowhead) in DB **F.** uPAR in DB was detected in endothelial cells (arrows) and stromal cells (arrowheads). **B-F.** Bar = 50 μm.
(see colour figures supplement)

The total staining indices in DSE were comparable to those in DP with only a higher total SI for MT1-MMP in DP (Table 3, $p<0.05$). Secondly, when DSE and DB were compared, only an elevated total SI for uPA was found in DB compared to DSE (Table 3, $p<0.05$). Thirdly, DB was compared to DP and this showed a similar pattern; only an elevated total SI for uPA in DB compared to DSE (Table 3, $p<0.05$).
Analysis of the protein expression in the various cell types showed that the mean staining indices were most abundant in glandular epithelial cells, followed by perivascular smooth muscle and stromal cells (Table 3, Figure 1). Endothelium displayed weak ex-

pression of all proteins, except MT5-MMP. MT5-MMP was not detectable (Figure 2). Significant differences were not found between the three decidual tissues with regard to

Table 3. First-trimester protein expression in early versus late first-trimester pregnancies [a].

		EC[b]		PSMC[b]		GE[b]		SC[b]		EVT[b]		Total SI[c]	
		early first-	late first-	early first-	late first-	early first-	late first-	early first-	late first-	early first-	late first-	early first-	late first-
MT1	**DSE**	0.6	0.0*	1.8	0.3	4.0	2.0	0.6	0.2	NP	NP	**7.0**#	**2.5***
	DP	0.7	0.0*	2.2	0.3	4.8	2.5	1.5	0.2	NP	NP	**9.2**	**3.0***
	DB	0.6	0.0*	1.7	1.0	3.7	3.0	1.7	1.2	1.1	0.5	**8.8**	**5.7**
MT2	**DSE**	1.3	0.5	3.4	0.7	4.2	3.5	1.1	0.0	NP	NP	**10.0**	**4.7***
	DP	1.0	0.3*	3.7	1.5	4.3	2.7	1.8	0.5	NP	NP	**10.8**	**5.0***
	DB	0.7	0.4	2.3	1.0	3.7	3.8	1.7	2.2	1.0	1.3	**9.4**	**8.7**
MT3	**DSE**	0.5	0.0	2.2	0.7	4.8	1.0	0.3	0.0	NP	NP	**7.8**	**1.7***
	DP	0.1	0.0	2.4	0.7	4.9	1.0	1.5	0.0	NP	NP	**8.9**	**1.7***
	DB	0.0	0.0	1.6	1.0	5.2	1.0	1.7	0.0	1.2	0.0	**8.8**	**2.0***
MT5	**DSE**	0.0	0.0	0.3	0.0	1.7	2.0	0.2	0.2	NP	NP	**2.2**	**2.2**
	DP	0.0	0.0	0.3	0.0	1.6	2.2	0.2	0.5	NP	NP	**2.1**	**2.7**
	DB	0.0	0.0	0.2	0.0	1.5	1.8	0.3	0.3	0.1	0.3	**2.1**	**2.4**
uPA	**DSE**	0.2	0.1	1.8	2.0	4.4	3.9	1.8	1.5	NP	NP	**8.2**	**7.5**
	DP	0.3	0.3	1.8	1.7	4.4	4.1	2.4	2.6	NP	NP	**8.9**	**8.7**
	DB	0.4	0.5	2.1	2.0	5.4	5.3	4.7	5.5	1.3	1.1	**13.9**$	**14.4**
uPAR	**DSE**	0.3	0.5	0.9	1.8	5.6	3.8	1.8	1.5	NP	NP	**8.6**	**7.6**
	DP	0.2	0.0	0.7	1.7	5.5	4.3	1.6	1.7	NP	NP	**8.0**	**7.7**
	DB	0.4	0.8	0.7	2.2	5.4	5.0	2.7	2.5	0.9	1.2	**10.1**	**11.7**

[a] 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 within groups and the general linear model was used to analyse differences between the two groups.

[bc] 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 endothelial cells (EC), perivascular smooth muscle cells (PSMC), glandular epithelium (GE), stromal cells (SC) and extra-villous trophoblasts (EVT) per decidual tissue.

NP = not present. # $p<0.05$ in DSE versus DP and $ $p<0.05$ in DB versus DP and DSE in the early first-trimester group. * $p<0.05$ in late vs early first-trimester decidua.

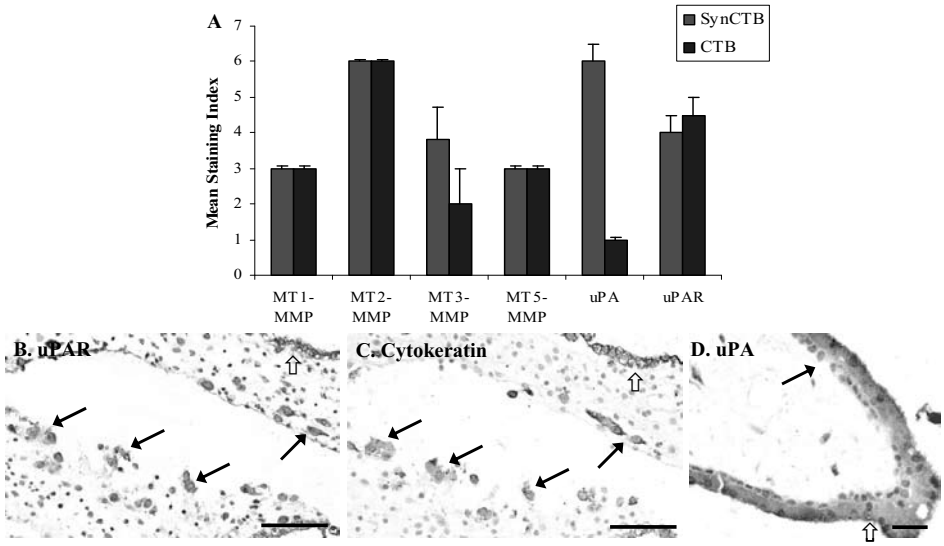

Figure 3. Protein levels of proteases in villous and extra-villous trophoblasts (EVT) of early first-trimester decidua.

The presence of protease antigens in villous and extravillous trophoblasts in decidua basalis (DB) was studied in serial sections stained against cytokeratin and the target protein. **A.** Immunostaining in syncytio- and cytotrophoblasts (synCTB and CTB) was expressed as the mean staining index ± SEM. **B.** and **C.** uPAR and cytokeratin antigens in serial expression in EVT (closed arrows) and epithelium (open arrows). **D.** uPA antigens in syncytiotrophoblast (open arrow) and not in cytotrophoblast (closed arrow). **B-D.** Bar = 50 μm.

(see colour figures supplement)

endothelial protein expression. 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 3, Figure 1 and 2).

All proteases studied were present in extra-villous trophoblasts, although MT5-MMP stained only dimly. Furthermore, all proteases were detectable in villous trophoblasts; uPAR, MT1-, MT2-, MT3- and MT5-MMP were moderately expressed in both syncytiotrophoblasts as cytotrophoblasts. The expression of uPA was weak in cytotrophoblasts, while it was abundantly present in syncytiotrophoblasts (Table 3, Figure 3).

Proteases in early versus late first-trimester decidua

The mRNA levels of proteases in late first-trimester decidua were compared to those in early first-trimester decidua (100%, Table 4). 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 to early first-trimester pregnancies. Comparison of DP between both groups showed elevated mRNA levels of MT1-MMP

Table 4. mRNA expression in late first-trimester pregnancies compared to early first-trimester pregnancies (100%).[a]

	Late 1st DSE[b]	Late 1st DP[b]	Late 1st DB[b]
MT1-MMP	206 %*	215 %*	150 %
MT2-MMP	1047 %*	80 %	110 %
MT3-MMP	38 %#	23 %#	92 %
MT5-MMP	111 %	98 %	107 %
uPA	131 %	205 %	168 %
uPAR	160 %	207 %*	137 %

[a] 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.

[b] The 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<0.02, # p<0.05

(215%, p<0.02) and uPAR (207%, p<0.02) and reduced mRNA expression 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 4).

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 3, p<0.05).

The mean SI's 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 expression was not even detectable in late first-trimester decidua. uPA and uPAR displayed comparable staining indices in endothelial cells at the two time points, while MT5-MMP was absent in these cells in both conditions (Table 3).

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 stromal cells.

The CD56 staining showed inhomogeneous staining, mainly localised 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 stromal cells. Both DP and DB showed a significantly larger number of CD56+ cells then DSE (9 ± 2%, p<0.05). No significant differences were detected between early and late first-trimester decidua (Figure 4 A and C).

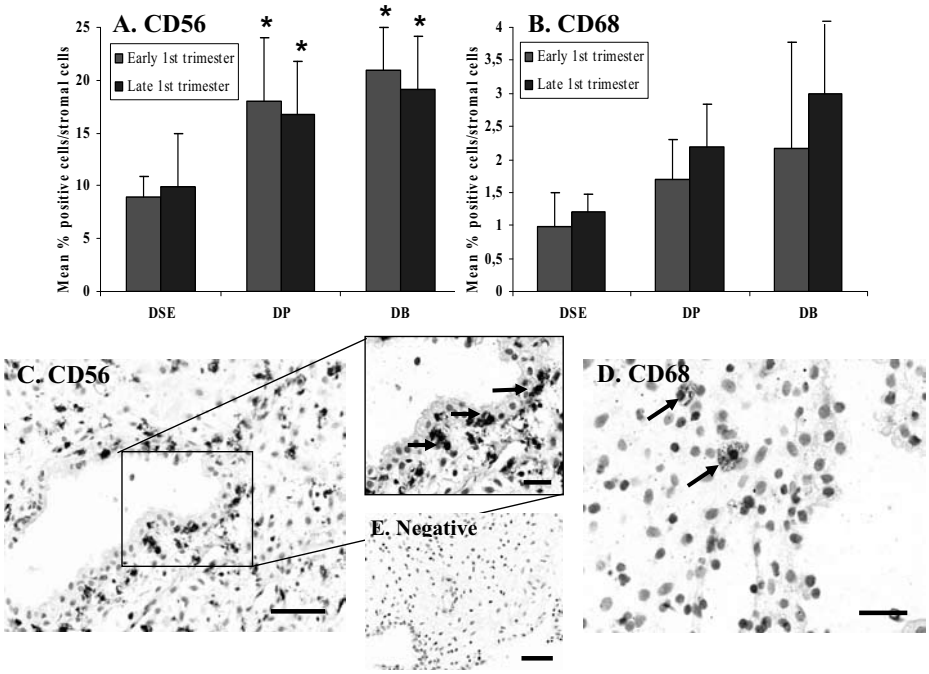

Figure 4. CD56- and CD68- positive cells in early and late first-trimester decidua.
A. CD56-positive cells in decidua, expressed as the mean number of positive cells per total number of stromal cells (mean ± SD) **B.** CD68-positive cells in decidua, expressed as the mean number of positive cells per total number of stromal cells (mean ± SD). **C.** CD56 expression in decidua basalis, bar = 100 μm. Blow up shows CD56+ cells (arrows) surrounding a vessel and gland, bar = 50 μm **D.** CD68-positive cell (arrow) in decidua parietalis, bar = 50 μm. **E.** Negative control, bar = 100 μm. * p<0.05 versus DSE
(see colour figures supplement)

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 (Figure 4 B and D).

DISCUSSION

The present study demonstrates that the mRNA and protein content of the pericellular-acting MT-MMPs and uPA/uPAR varied between decidual secretory endometrium (DSE), decidua basalis (DB) and decidua parietalis (DP). Furthermore, the percentage of CD56-positive cells was two-fold lower in DSE than in DB and DP. A comparable increase in macrophages did not reach statistical significance. It is likely that the differential expression of the proteases is mainly due to the different effects of pregnancy-

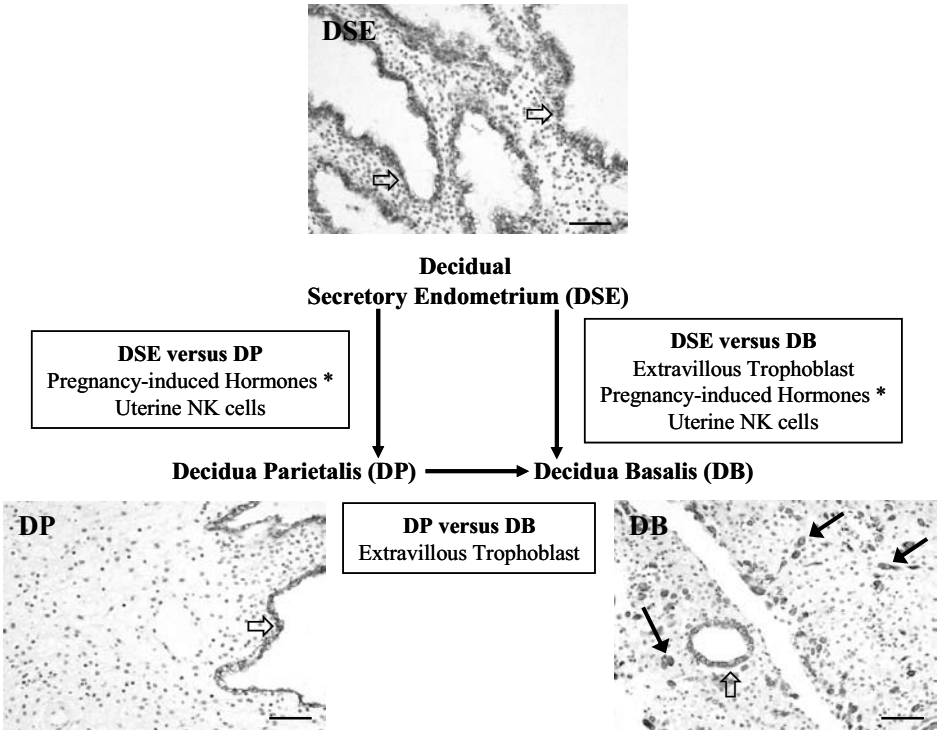

Figure 5. Human first-trimester decidual tissues.
Representation of the influences of immune cells, extra-villous trophoblast and pregnancy-induced hormones (*hCG, oestradiol and progesterone) on first-trimester decidual tissues. Differentiation between decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB), was obtained by HPS and cytokeratin staining. DSE and DP express cytokeratin in glandular epithelium (open arrows), whereas cytokeratin is also expressed in EVT (closed arrows) of DB. Bar =100 μm.
(see colour figures supplement)

induced hormones (hCG, oestradiol and progesterone), the extra-villous trophoblast 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 expression 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, oestradiol and progesterone, immune cells, and the extra-villous trophoblasts (EVT). Pregnancy-induced hormones are involved in the development of decidua parietalis (DP) from the decidual secretory endometrium (DSE), while the additional presence of the EVT induces the generation of the decidua basalis (DB). This may imply that

differences in protease expression 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-Truijllo *et al.,* 2003]. As these uNK cells are known to produce many chemokines and angiogenic growth factors [Hanna *et al.,* 2006;Li *et al.,* 2001a; 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, 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 percentual contribution of uNK cells and macrophages to the immune cell and overall stromal cell populations were comparable with those reported by other investigators [Tuckerman *et al.,* 2007; Quenby *et al.,* 1999; Bulmer *et al.,* 1991]. Interestingly, the amounts of uNK cells were twofold 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 stromal cells). To our knowledge the difference between DSE and DB/DP has not been systematically investigated before.

The foregoing information is summarised in Figure 5, which presents a model that describes the influences of uNK cells, extra-villous trophoblast 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 extra-villous trophoblast [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 decidua basalis and parietalis (DB and DP) as compared to decidual secretory endometrium (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 [Hurskainen *et al.,* 1998; Nawrocki *et al.,* 1996; Tanaka *et*

al., 1998]. Finally, the fact that MT1-MMP is induced in various cell types of decidua parietalis and basalis also points to a role in decidual remodelling and/or vascularisation.
MT2-MMP and MT3-MMP mRNA levels were increased and MT5-MMP mRNA expression was 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 perivascular smooth muscle cells 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 vascularisation pattern in serial sections of the same specimens, which showed highly enhanced vascularisation at the implantation site. Comparison of these data with the endothelial expression of MT2- and MT3-MMPs shows that the amounts of these endothelial MT-MMPs correlated well with the number of vascular structures/mm^2 and correlate inversely with the luminal surface of these vascular structures [Plaisier *et al.,* 2007]. Furthermore, experiments with endometrial endothelial cells in vitro indicated that MT2- and MT3-MMP are potential candidates for regulation of endometrial angiogenesis [Plaisier *et al.,* 2006; Plaisier *et al.,* 2007]. These data leads us to hypothesise that MT2-MMP and MT3-MMP play a role in vascular adaptation to pregnancy at the implantation site.

UPA and uPAR were detected in the various decidual cell types. In contrast to cycling secretory endometrium, in which endothelial and stromal cells contained uPA antigen and glandular epithelium 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 stromal cells. The expression of uPAR largely follows the pattern of uPA and is clearly present in endothelial cells. The presence of uPAR in EVT is confirmed by previous studies and suggests a role in trophoblast invasion [Floridon *et al.,* 1999; Pierleoni *et al.,* 1998,].
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 downregulation of uPA mRNA by hCG, an important product of trophoblasts [Salamonsen 1999; Yagel *et al.,* 1993]. However, uPA was increased at the protein level in all cell types in DB compared to 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 internalises uPA in complex with its inhibitor PAI-1 [Blasi, Carmeliet 2002; Kroon *et al.,* 1999]. Furthermore, uPA and uPAR are present in a part of

the stromal cells 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 first-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 matrix metalloproteinases 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 to early first-trimester pregnancy.

Furthermore, the MT1- MT2- and MT3-MMP antigen expression in endothelium and perivascular smooth muscle cells was also reduced in all types of decidua of late first-trimester pregnancy. We recently determined the vascularisation pattern in serial sections of the same early and late first-trimester decidua samples and this showed that vascularisation is regulated as gestation progresses. The endothelial expression of MT1-, MT2- and MT3-MMPs correlated well with the differences in vascularisation 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 neovascularisation 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 decidual secretory endometrium (DSE), decidua basalis (DB) and decidua parietalis (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 hypothesising 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 vascularisation (Figure 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 extra-villous trophoblast. 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 during

implantation 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 decidualisation may contribute to new insights in currently non-explicable pathological events associated with pregnancy, such as miscarriages and pre-eclampsia.