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

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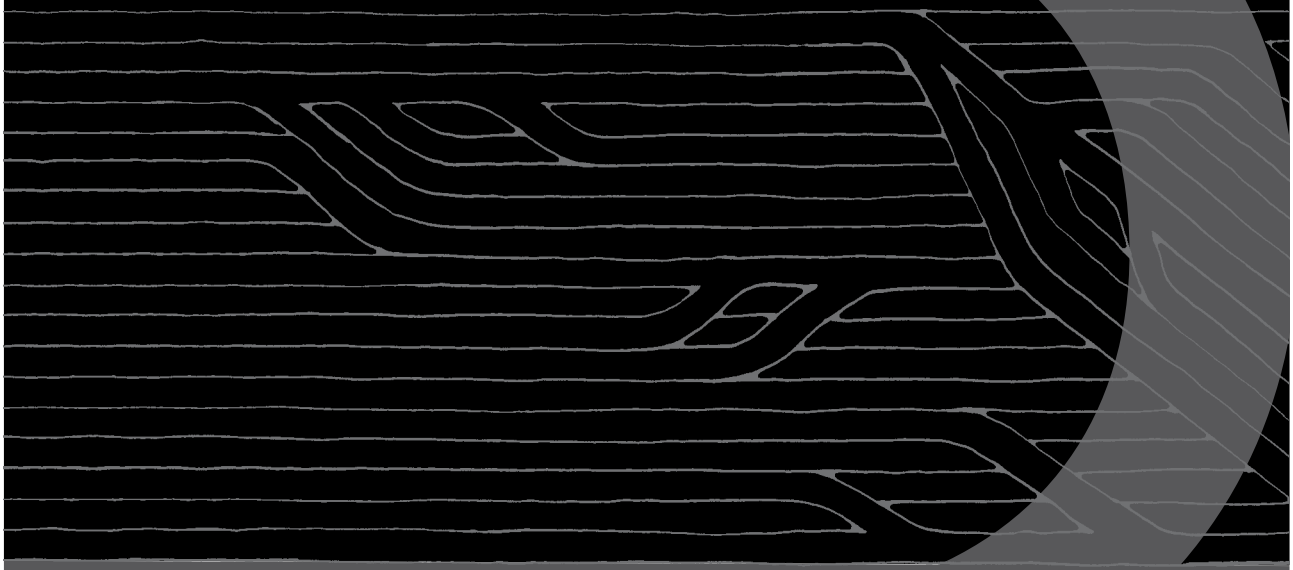
Chapter 5

Different degrees of vascularisation and their relationship to the expression of VEGF, PlGF, Angiopoietins and their receptors in first-trimester decidual tissues.



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ABSTRACT

Objective: To evaluate vascular adaptation to implantation by studying vascularisation and angiogenic factors in decidua basalis (DB), decidua parietalis (DP) and decidual secretory endometrium (DSE) of first-trimester pregnancies. Comparing these tissues provides information about the regulation of vascularisation by pregnancy-induced hormones and/or the extra-villous trophoblast (EVT).

Patients & Methods: Decidual samples from vacuum-aspiration were collected from women (n=32) undergoing voluntarily first-trimester termination of pregnancy. Vascularisation, determined by CD34-immunohistochemistry, and VEGF-A, PlGF, Flt-1, KDR, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and TIE-2 protein and mRNA expression, determined by RT-PCR and immunohistochemistry, were evaluated in serial paraffin sections.

Results: Pregnancy-induced hormones and EVT influence vascularisation by enhancing vascular and luminal surface and reducing vessel density at the implantation site. These changes correlate with differences in gene and protein expression. PlGF mRNA and PlGF and Flt-1 protein expressions were elevated in DB under influence of EVT. Also the angiopoietins were differentially expressed, in favor of Ang-2, in DB.

Conclusions: The EVT and pregnancy-induced hormones regulate vascularisation and the expression of angiogenic factors in decidua. The induction of PlGF, Flt-1, and Ang-2/Ang-1 ratio in DB suggests that these factors play a role in regulating angiogenesis at the implantation site.

INTRODUCTION

Vascularised, receptive endometrium is essential for implantation and for the success of the embryo-maternal interaction [Zygmunt *et al.,* 2003]. Disturbances in vascular development may play an important role in frequently occurring pathologies during pregnancy, such as early pregnancy wastage, pre-eclampsia and foetal growth restriction [Vailhe *et al.,* 1999; Vuorela *et al.,* 2000; Zygmunt *et al.,* 2003].
Endometrial adaptation to implantation starts during the menstrual cycle by means of stromal decidualisation and the induction of angiogenesis, the formation of new vessels from existing vasculature. These processes continue after conception under influence of oestradiol, progesterone, hCG, cytokines and growth factors derived from corpus luteum, endometrium and implanting embryo.
Angiogenic changes of the decidua include proliferation, migration and increased vascular permeability [Goodger, Rogers 1993; O'Shea *et al.,* 1983]. After the 6th week of gestation, the arterioles are remodelled from low-capacitance high-resistance to high-capacitance low-resistance vessels, and by the 12th week maternal blood flow to the intervillous space is established [Burton *et al.,* 1999; Pijnenborg *et al.,* 1983].

In general, angiogenesis is a multi-step process [Carmeliet 2003; Ferrara 2000; Plaisier *et al.,* 2004; Smith 2000]. Numerous factors are able to regulate angiogenesis, of which the vascular growth factors, Vascular Endothelial Growth Factor-A (VEGF-A) and Placental Growth Factor (PlGF), are probably the most well known. VEGF interacts with the VEGFR-1 (Flt-1) and VEGFR-2 (KDR) to promote endothelial cell proliferation, cell migration and vascular permeability. In rabbits, VEGF, together with its receptor Flt-1, plays an active role in the angiogenic process during implantation [Das *et al.,* 1997a].
PlGF shares biochemical and functional features with VEGF and interacts with VEGFR-1 (Flt-1). PlGF and VEGF have synergistic effects regarding angiogenesis, but vessels induced by PlGF are more mature and stable than vessels induced by VEGF [Carmeliet 2003; Luttun *et al.,* 2002]. PlGF is abundantly expressed in human placenta and may be an important paracrine regulator of decidual angiogenesis and an autocrine mediator of trophoblast function [Sherer, Abulafia 2001].

A second family of growth factors, the angiopoietins, and their receptor TIE-2 are also known for their regulating capacities regarding angiogenesis. The two ligands bind with equal affinity to TIE-2 but have different functions. Angiopoietin-1 maintains vessel integrity and plays a role in the later stages of vascular remodelling [Geva, Jaffe 2000]. Transgenic overexpression of Ang-1 in mice results in the development of more complex vascular networks [Suri *et al.,* 1998].

Angiopoietin-2 is a functional antagonist of Ang-1 and leads to loosening of cell/cell interactions and allows access to angiogenic inducers like VEGF [Maisonpierre *et al.,* 1997]. Co-expression of VEGF and Ang-2 induces angiogenesis, but Ang-2 results in vascular regression in the absence of angiogenic signals [Asahara *et al.,* 1998]. Ang-1 is widely expressed in the adult, whereas Ang-2 is selectively expressed at sites of active angiogenesis, such as ovary, uterus and placenta [Davis *et al.,* 1996; Maisonpierre *et al.,* 1997].

Vascular adaptation to implantation and its regulation have been studied thoroughly with regard to arterial remodelling and placental vascularisation [Craven *et al.,* 1998; Kam *et al.,* 1999]. However, it is unknown how maternal decidual vascularisation is regulated and whether regulatory signals are originating from the extra-villous trophoblast and/or from pregnancy-induced hormones. Therefore, the present study was performed in three first-trimester decidual tissues: decidual secretory endometrium (DSE), decidua basalis (DB) and parietalis (DP).
By comparing these decidual tissues within subjects, the changes that occur independently of trophoblast invasion, i.e. pregnancy-induced hormones, can be separated from the influence of the invasive extra-villous trophoblast (EVT, Fig. 1). Vascularisation pattern and the expressions of VEGF-A, PlGF, Flt-1, KDR, Ang-1, Ang-2 and TIE-2 were determined in these three decidual tissues. In this way, decidual vascular adaptation to embryonic implantation can be studied in detail.

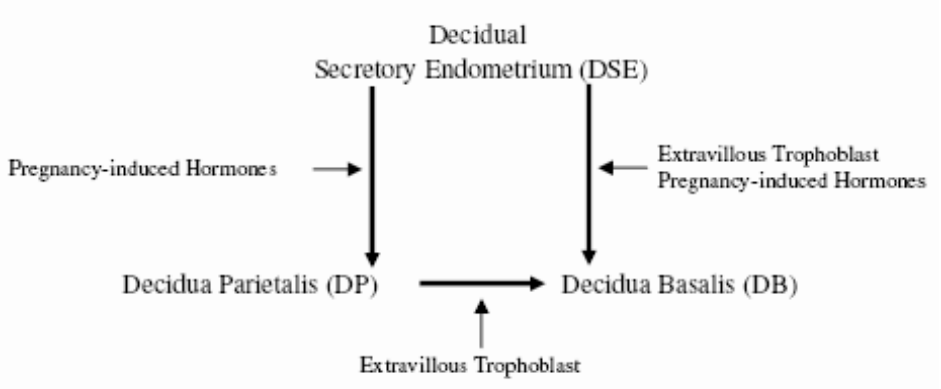

Figure 1. Schematic representation of the influences of extra-villous trophoblast and pregnancy-induced hormones on decidual tissues of first-trimester pregnancies.

MATERIALS AND METHODS

Materials

Lysis buffer (Tris 20 mM pH 7.4, EDTA 1 mM pH 8.0, 2% SDS), 20 mg/ml proteinase K (Life-Technologies Gibco BRL, Gaithersburg MD) and solution D (4 M guanidium isothiocyanate, 0.75 M Sodiumcitrate, 10% sarkosyl and 2-mercapto-ethanol) were used for RNA isolation. cDNA synthesis was performed using Ready-to-go You-Prime first strand beads (Amersham Biosiences, Buckinghamshire, UK). Primer and probe (FAM/TAMRA double-labelled) sets for VEGF-A, VEGF-R1 (Flt-1), VEGF-R2 (KDR), PlGF, Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), TIE-2 and GAPDH (VIC-labelled) were purchased from Applied Biosystems (Foster City, CA USA).

The following first antibodies were used: broad spectrum polyclonal rabbit anti-cytokeratin (1:2000, Z0622, DAKO, Glostrup Denmark), monoclonal mouse anti-CD34 (1:1000, QBend/10, Novocastra, Newcastle upon Tyne, UK), polyclonal rabbit anti-VEGF-A (1:100, sc-152, Santa Cruz, CA, USA), polyclonal rabbit anti-VEGFR-1 (Flt-1, 1:50, sc-316, Santa Cruz, CA, USA), monoclonal anti-VEGFR-2 (KDR, 1:50, sc-6251, Santa Cruz, CA, USA), polyclonal goat anti-PlGF (1:200, sc-1880, Santa Cruz, CA, USA), polyclonal goat anti-Angiopoietin-1 (1:50, sc-6319, Santa Cruz, CA, USA), polyclonal goat anti-Angiopoietin-2 (1:50/, sc-7016, Santa Cruz, CA, USA) and polyclonal rabbit anti-TIE-2 (1:100, sc-9026, Santa Cruz, CA, USA).

The following second 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), biotinylated rabbit anti-goat (1:300, E-0466, DakoCytomation, Glostrup, Denmark). Immunohistochemical reactions were visualised using avidin-biotin complex (DakoCytomation, Glostrup, Denmark) and NovaRED (Vector, Burlingame, USA).

Study group

Decidua samples were obtained from women (n=32) with a healthy, viable intrauterine gravidity, undergoing vacuum aspiration due to a legal voluntarily abortion. The study was approved by 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 gestational age (GA). We created two distinct groups with mean GA<8-9 weeks and GA>8-9 weeks. This cut-off of GA 8-9 weeks was chosen because evidence exists that this period is associated with changes in placental vascu-

Table 1. Characteristics of the study subjects.

	Group I (*n*=25) Mean ± SD	**Group II** (*n*=8) Mean ± SD
Maternal Age (years)	29.4 ± 6.9	29.6 ± 14.4
Gestational age (days)	45.3 ± 7.8	90.1 ± 12.6
Gestational age (weeks)	6.5 ± 1.1	12.6 ± 1.8
Number of pregnancies	1.3 ± 0.3	1.6 ± 0.5
Number of Miscarriages	0.2 ± 0.1	0.4 ± 0.3

larisation [Burton *et al.,* 1999]. After 8-9 weeks of GA, the intervillous space is gradually filled with maternal blood, causing oxygen level and oxidative stress to rise, thereby stimulating placental differentiation and vascularisation. This process is thought to be largely completed at 12 weeks gestation. This cut-off was the basis of the early first-trimester group (n=25) with a mean GA of 45.3 days (± 7.8) and the late first-trimester group (n=8) with a mean GA of 90.1 days (± 12.6). 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, fixed in formaldehyde and embedded in paraffin. A Hematoxilin Phloxin Safrane (HPS) staining was used to differentiate between decidua and decidual secretory endometrium (DSE), which microscopically resembles secretory endometrium during the menstrual cycle. Decidua basalis (DB) and decidua parietalis (DP) were differentiated by the presence or absence of extra-villous trophoblasts using an anti-cytokeratin staining (Fig. 2). Only subjects with a complete set of DSE, DB and DP were included. Serial sections of the paraffin embedded tissue samples were used for all experiments and all outcome measures were compared between tissues within subjects.

RNA isolation and cDNA synthesis

RNA was extracted from paraffin embedded tissue samples based on methods described previously [Specht *et al.,* 2001]. 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 and DB (without villous tissue), were macro-dissected and dissolved in 190 μl lysis buffer and 10 μl proteinase K for 18 hours at 60°C. Subsequently, 400 μl Solution D was added and RNA was isolated according to the Chomczynski method [Chomczynski, Sacchi 1987]. RNA quantity and quality was determined by measuring its absorbance in a

spectrophotometer (Nanodrop ND-1000) and reverse transcription was performed with 1 μg total RNA, random primers and a cDNA synthesis kit.

Real-time RT-PCR

The mRNA expression was quantified according to the Taqman real-time PCR method using validated primer and probe (FAM/TAMRA double-labelled) sets for VEGF-A, VEGF-R1 (Flt-1), VEGF-R2 (KDR), PlGF, Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), TIE-2. glycerylaldehyde-3-phosphate dehydrogenase (GAPDH, primers/VIC-labelled probe) was used as an endogenous reference gene. 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 duplicate, expressed in cycle threshold (Ct) and quantified into ng/μl using a standard curve of total RNA. The amount of RNA expression of 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 trypsin was used for the detection of TIE-2, whereas detection of cytokeratin, Ang-2 and Ki67 required boiling in 0.1 M citrate buffer. Primary antibodies were applied overnight at 4°C followed by incubation with a biotinylated secondary antibody. Antibody binding was visualised using avidin-biotin and streptavidin-horseradish peroxidase conjugates. Sections were counterstained with Mayer's hematoxylin. Specificity of the immunohistochemical reaction was verified by the omission of the first antibody as well as using normal mouse, goat or rabbit serum as 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 the vascularisation pattern

CD34-stained endometrial sections were used to scan sequential fields. Two to four samples of each type of tissue were analysed of each study subject and six fields per sample were scanned at 100x magnification. The number of vessels per mm^2, vascular surface per mm^2 (mm^2/mm^2) and luminal surface (μm^2/vessel) were determined using image analysis software (Qwin, Leica Microsystems).

Evaluation of Immunohistochemical staining

Immunostaining was evaluated by calculating a staining index (SI): proportion of stained cells x staining intensity. 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 population. The intensity of staining was expressed as 1, 2 or 3 (weak, moderate or strong staining, respectively). The minimum score was 0 and the maximum score 9 [Nap *et al.,* 2004]. 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), peri-vascular 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.

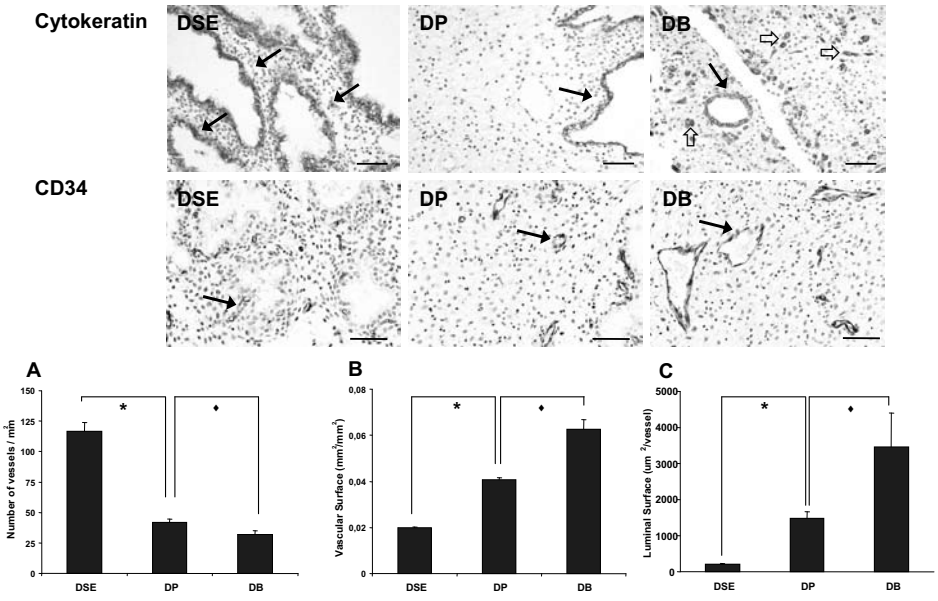

Figure 2. Cytokeratin expression and vascularisation in the three decidual tissues.
Differentiation between three decidual tissues was obtained by HPS and anti-cytokeratin staining (top **panel**). DSE and DP expressed cytokeratin only in glandular epithelial cells (closed arrows), whereas cytokeratin is also expressed in extra-villous trophoblasts of DB (open arrows). The vascularisation pattern in human decidual tissues was determined by image analysis of anti-CD34-stained sections (**middle and bottom panel**). CD34 antigen expression in endothelial cells (arrows) of DSE, DP and DB was compared within subjects. Data were analysed using the repeated measures ANOVA. **A.** The number of vessels per mm², **B.** the vascular surface per area (mm²/mm²) and **C.** the luminal surface (μm²/vessel) were calculated and expressed as mean ± SEM. * p<0.0001; ♦ p< 0.04. Bar =100 μm.
(see colour figures supplement)

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). 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

Vascularisation in early first-trimester decidua

Intense CD34 staining was observed in all vessels of the three decidual tissues, with no difference in staining intensity between the tissues.

The vessel density was significantly elevated in DSE (116.4 vessels/mm^2) compared to DP (41.7 vessels/mm^2, $p<0.001$) and DB (31.8 vessels/mm^2, $p<0.0001$); vessel density in DP and DB also differed significantly ($p<0.03$, Fig 2A). Since the stromal cells concentration differed between the tissues (434 cells/ mm^2 in DSE, 208 cells/mm^2 in DB and DP, $p<0.05$), vessel density was corrected for the stromal cell count. The corrected vessel density showed a similar pattern; density was elevated in DSE (0.28 vessels/ stromal cells) compared to DP and DB and elevated in DP (0.20 vessels/stromal cells) compared to DB (0.15 vessels/stromal cells).

The vascular surface was significantly smaller in DSE (0.020 ± 0.0001 mm^2/mm^2) compared to DP (0.041 ± 0.001 mm^2/mm^2) and DB (0.063 ± 0.004 mm^2/mm^2, all $p<0.0001$, Fig. 2B). The vascular surface in DP differed significantly from that in DB ($p<0.04$).

DSE showed a significant smaller luminal surface in comparison with DP (208 versus 1474 μm^2/vessel, $p<0.0001$) and DB (3465 μm^2/vessel, $p<0.001$); this parameter was also significantly smaller in DP compared to DB ($p<0.05$, Fig 2C).

Thus, pregnancy-induced hormones and extra-villous trophoblasts influence vascularisation, since larger but fewer vessels were present in DP compared to DSE and in DB compared to DP (Fig. 1 and 2).

mRNA levels of angiogenic factors

The expression of several angiogenic growth factors and their receptors were evaluated at the RNA level (Table 2). All tested genes were expressed by the three decidual tissues. First, the mRNA levels of the angiogenic factors in DSE were compared with those in DP. Only TIE-2 was differentially expressed, reflected by a 49% reduction in DP compared

Table 2. Differential gene expression in early first-trimester decidua [a].

	DSE[b]	DP	DSE[b]	DB	DP[c]	DB
PlGF	100 %	82 ± 21%	100 %	1286 ± 302%*	100 %	3000 ± 237%[$]
VEGF-A	100 %	85 ± 12%	100 %	96 ± 14%	100 %	134 ± 36%
KDR	100 %	105 ± 16%	100 %	74 ± 15%	100 %	76 ± 14%
Flt-1	100 %	105 ± 14%	100 %	130 ± 24%	100 %	131 ± 23%
Ang-1	100 %	92 ± 17%	100 %	39 ± 11%*	100 %	55 ± 15%[$]
Ang-2	100 %	115 ± 29%	100 %	34 ± 8%*	100 %	36 ± 6%[$]
TIE-2	100 %	51 ± 11%[#]	100 %	21 ± 5%*	100 %	43 ± 7%[$]

[a] RNA levels were determined by RT-PCR in DSE, DP and DB of early first-trimester (n=25) and compared within subjects. The 'repeated measures ANOVA' was performed to analyse the data.
[b] The RNA levels of DSE was set at 100% to compare mRNA levels in DSE with those in DP and DB.
[c] The RNA levels of DP was set at 100% to compare DP with DB.
[#] $p < 0.02$ DP compared to DSE, * $p < 0.02$ DB compared to DSE, [$] $p < 0.01$ DB compared to DP.

to DSE ($p<0.02$). The other genes, VEGF-A, PlGF, Flt-1, KDR, Ang-1 and Ang-2, showed comparable mRNA levels in DP and DSE (Table 2).
Subsequently, the mRNA levels in DSE were compared to those in DB. This comparison showed a 1286 % induction of PlGF, 61% reduction of Ang-1, 66% reduction of Ang-2 and a 79% reduction of TIE-2 in DB compared to DSE (Table 2, $p<0.02$). The expressions of VEGF, Flt-1 and KDR did not differ significantly. Finally, mRNA expressions in DB were compared to that in DP to evaluate the influence of the extra-villous trophoblast (EVT). This comparison demonstrated a similar pattern as the comparison DSE to DB, namely an increased expression of PlGF (3000%) and reduced expressions of Ang-1 (45%), Ang-2 (64%) and TIE-2 (57%) in DB compared to DP (Table 2, all $p<0.01$).

The expression of angiogenic factors at protein level of early first-trimester decidua

The distribution of angiogenic factors was determined at the protein level in serial sections of DSE, DP and DB. The studied proteins were detectable in all decidual tissues (Table 3).
The protein expressions in DSE were compared to those in DP, which only showed a higher total staining index for Flt-1 in DP (Table 3, $p<0.05$). In addition, when DSE and DB were compared, higher endothelial and total expressions of both PlGF and its receptor Flt-1 were demonstrated in DB. The total Ang-1 expression was lower in DB (Table 3, $p<0.05$). Thirdly, DB was compared to DP and this showed a similar pattern as the comparison DSE and DB, namely higher total and endothelial expressions of PlGF and Flt-1 in DB (Table 3, Fig. 3A, C and E, $p<0.05$). Also, the total Ang-1 expression was less abundant in DB ($p<0.01$), which resulted in a higher Ang-2/Ang-1 ratio in DB than in the other tissues (2.6 vs.1.4 and 1.2, $p<0.01$). The expressions of VEGF-A, KDR, Ang-2 and TIE-2 proteins were comparable in the three tissues.

Table 3. Protein expression of angiogenic factors in early first-trimester decidua [a].

		EC[b]	PSMC[b]	GE[b]	SC[b]	EVT[b]	Total SI[c]
VEGF	**DSE**	0.1	3.5	5.3	1.7	NP	**10.6**
	DP	0.3	3.3	6.2	3.0	NP	**13.1**
	DB	0.0	2.5	4.9	2.6	3.3	**13.3**
PlGF	**DSE**	0.1	3.4	5.4	2.5	NP	**11.4**
	DP	0.1	2.4	4.8	3.0	NP	**10.3**
	DB	0.9 *	2.7	6.2	3.3	2.8	**15.9** *
KDR	**DSE**	1.0	5.4	6.0	0.7	NP	**13.1**
	DP	0.6	3.5	4.7	2.2	NP	**11.0**
	DB	0.9	3.2	4.4	2.0	0.8	**11.3**
Flt-1	**DSE**	0.5	2.7	5.8	1.6	NP	**10.6** &
	DP	0.4	3.3	5.6	3.3	NP	**12.6**
	DB	1.2 *	3.4	5.4	2.5	2.2	**14.7** *
Ang-1	**DSE**	0.0	1.1	4.7	0.3	NP	**6.1**
	DP	0.0	1.3	4.4	0.9	NP	**6.6**
	DB	0.1	0.0	3.1	0.2	0.0	**3.4** *
Ang-2	**DSE**	0.2	1.7	5.6	0.0	NP	**7.5**
	DP	0.6	1.1	6.2	1.7	NP	**9.6**
	DB	0.7	1.5	4.9	0.9	0.9	**8.9**
TIE-2	**DSE**	1.8	3.2	6.0	2.2	NP	**13.2**
	DP	2.3	3.5	5.1	4.1	NP	**15.0**
	DB	1.8	2.1	4.1	3.1	2.8	**13.9**

[a] The protein expression of angiogenic factors in DSE, DP and DB were compared within subjects. Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis.
[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), peri-vascular 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 DB versus DP and DSE, & $P<0.05$ in DSE versus DP.

Endothelium displayed the expression of all angiogenic factors, except Ang-1 (Fig. 3A and F). VEGF-A was hardly detectable in endothelium (Fig. 3A and B). Ang-2 and KDR were mainly expressed by glandular epithelium and PSMCs, but also by endothelial cells (Fig.

3A, D and G). Moreover, TIE-2 showed the highest expression in endothelium (Table 3, Fig. 3A and H).

All angiogenic factors, except Ang-1, were also detected in extra-villous trophoblasts; PlGF, VEGF, Flt-1 and TIE-2 were moderately expressed, whereas KDR and Angiopoietin-2 expressions were weak (Table 3, Fig. 4).

Vascularisation and expression of angiogenic factors in late first-trimester decidua

The vascularisation parameters in late first-trimester decidua showed similarities with vascularisation in the early first-trimester; the vascular and luminal surface were larger and vessel density was smaller in DP and DB compared to DSE and in DB compared to DP (all $p<0.05$, Fig. 5A, B and C). Comparison of the vascularisation patterns at both time points showed that the vessel density was lower in DB and DP of late first-trimester

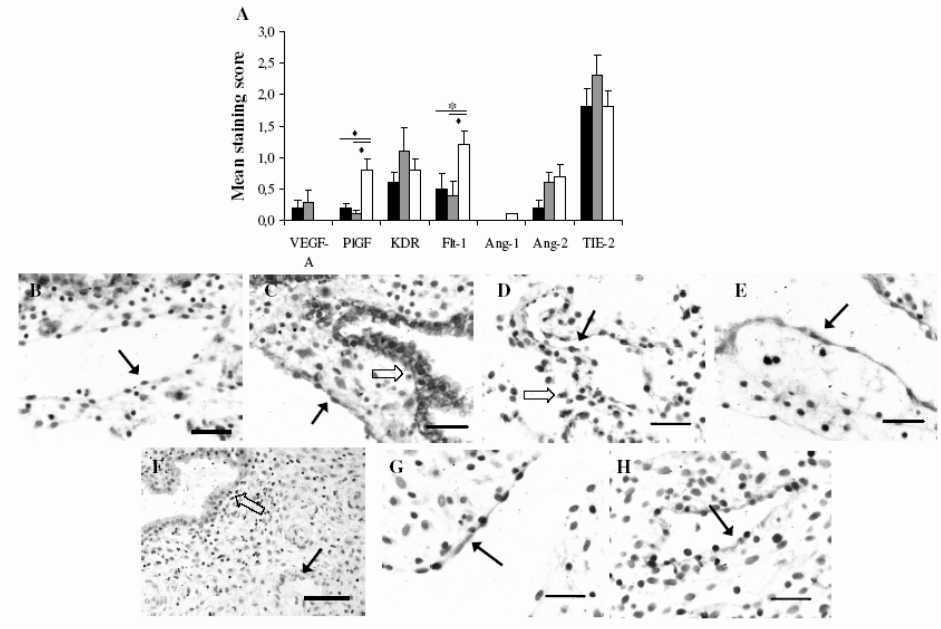

Figure 3. Protein expression of angiogenic factors in endothelium of early first-trimester decidua.
The protein expression of angiogenic factors was determined in DSE, DP and DB. Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis. **A.** Protein expression of each angiogenic factor in endothelium was expressed as the mean staining index ± SEM and **B-H** show examples of these expressions. **B.** Endothelial VEGF expression was not detectable in DB. **C.** PlGF was expressed in epithelium (open arrow) and endothelium (closed arrow) of DB. **D.** Endothelial KDR expression (closed arrow) and stromal KDR expression (open arrow) in DB. **E.** Endothelial Flt-1 expression in DB (arrow) **F.** Angiopoitein-1 in DB was detected in epithelium (open arrow), but not in stromal and endothelial cells (closed arrow). **G.** Endothelial angiopoietin-2 expression in DB (arrow). **H.** Endothelial TIE-2 expression in DB (arrow). ■ DSE, ■ DP, □ DB. Bar = 100 μm (except F = 50 μm).
(see colour figures supplement)

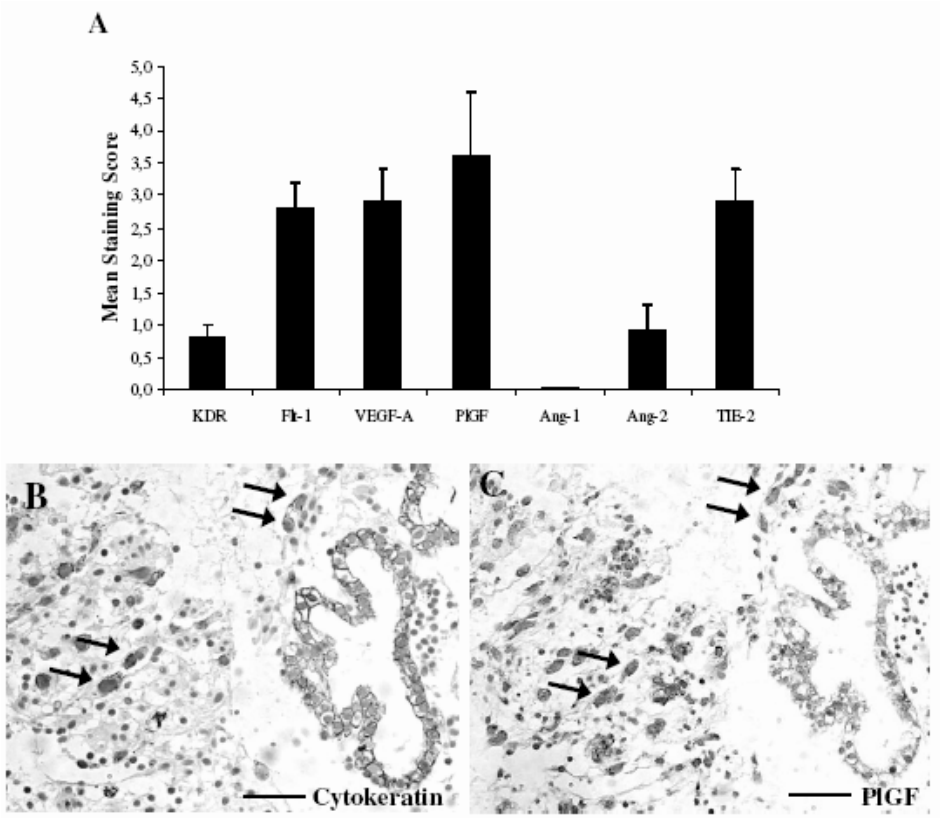

Figure 4. Protein expression of angiogenic factors in extra-villous trophoblasts (EVT) of early first-trimester decidua.
The protein expression of angiogenic factors in EVT in decidua basalis (DB) was studied in serial sections stained against cytokeratin and the target protein. **A.** Protein expression in EVT was expressed as the mean staining index ± SEM. **B.** Cytokeratin (arrows) and **C.** serial PlGF expression (arrows) in EVT. Bar = 50 um. (see colour figures supplement)

(17.8 vs. 31.8 vessels/mm^2 in DB and 29.2 vs. 41.7 vessels/mm^2 in DP, $p<0.05$). The luminal surface was larger, although not significantly, in DB and DP of late first-trimester, namely 5447 vs. 3465 μm^2/vessel in DB and 1829 vs. 1474 μm^2/vessel in DP (Fig.5B). The vascular surface was similar in decidua at the two time points (Fig. 5C).

Then, mRNA levels in early and late first-trimester were compared. VEGF-A and Ang-2 mRNA were both induced in late first-trimester DB and DP as compared to early first-trimester; the induction was 347% ($p=0.08$) in DB and 288% ($p<0.01$) in DP for VEGF-A and 257% ($p<0.01$) in DB and 171% ($p<0.01$) in DP for Ang-2. Genes in DSE showed no differential expression in time. At the protein level, total VEGF, Ang-1 and Ang-2 protein expressions were elevated in DB of late first-trimester compared to early first-trimester (165%, 288% and 174% respectively, $p<0.002$). Interestingly, VEGF and PlGF proteins

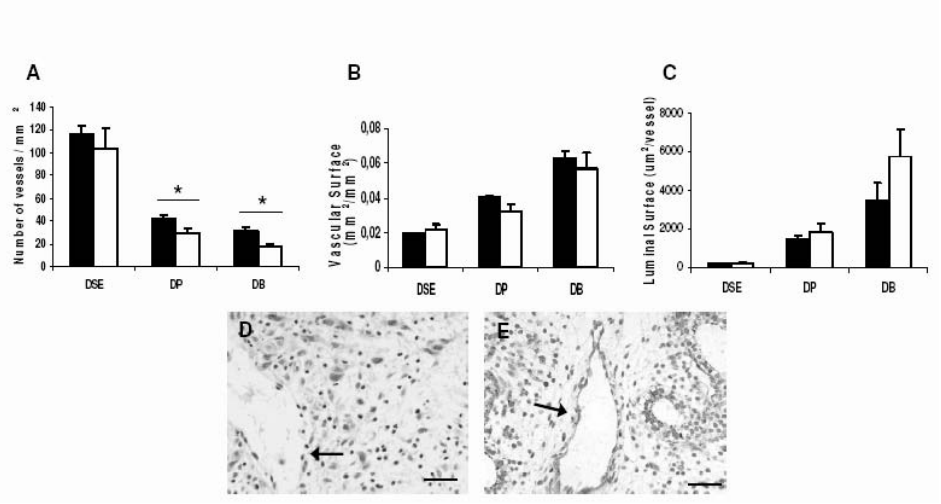

Figure 5. Vascularisation pattern and endothelial protein expression in early versus late first-trimester decidua.

The vascularisation pattern in human early (black bars) and late (white bars) first-trimester decidual tissues was determined by image analysis of anti-CD34-stained sections. Data were analysed using the repeated measures ANOVA. **A.** The number of vessels per mm^2, **B.** the vascular surface per area (mm^2/mm^2) and **C.** the luminal surface (μm^2/vessel) were expressed as mean ± SEM. VEGF expression was not detectable in early first-trimester endothelium of DB (arrow, **D**), whereas the expression was present in late first- trim. endothelium of DB (arrow, **E**). Early first-trimester and late first-trimester. Bar = 50 um, * $p<0.04$.
(see colour figures supplement)

were detected in higher amounts on the endothelium of late first-trimester decidua than in early first-trimester (Fig. 5D and E, $p<0.05$). Endothelial Flt-1, KDR, Ang-2 and TIE-2 expressions were comparable in early and late first-trimester.

DISCUSSION

The present study showed differences in vascularisation pattern between decidual secretory endometrium (DSE), decidua basalis (DB) and decidua parietalis (DP). These vascular changes correlated with differences in gene expression. Furthermore, analysis of vascularisation in early and late first-trimester decidua suggests that decidual vascularisation is remodelled as gestation progresses to meet up with the increasing needs of the implanting embryo.

Maternal vascular adaptation to implantation and its regulation has been subject to discussion, but has only been studied thoroughly with regard to arterial remodelling and villous vascularisation [Craven *et al.,* 1998; Kam *et al.,* 1999]. The present study focused on decidual vascular adaptation to implantation and its regulation. The comparison of three decidual tissues, DSE, DB and DP, within subjects provides the opportunity

to separate the influence of the extra-villous trophoblast (EVT) from the changes that occur independently of trophoblast invasion (Fig. 1). Furthermore, all methods were performed on serial paraffin sections, which allowed studying vascularisation, RNA and protein expression in the same tissue.

Changes in vascularisation pattern, the increase of vascular and luminal surfaces and decrease of vessel density, were detected between the three decidual tissues DSE, DP and DB. The enlargement of the vascular surface per area in DB and DP has been reported previously and likely is functionally related to the increased blood flow during pregnancy [Kam *et al.,* 1999; Gibbons, Dzau 1994]. The increase in luminal diameter may represent an additional modulatory effect, although the possibility that this was due to vasodilatation cannot be excluded. Increased luminal diameter in DP and DB could also be induced by a relatively low oxygen levels, but whether oxygen levels in DP, DB and DSE differ is not known [Charnock-Jones 2002].
The vessel density in decidual secretory endometrium (116 vessels/mm^2) is lower than the vessel density reported in cycling secretory endometrium, 186 vessels/mm^2 reported by Rogers *et al* and 195 vessels/mm^2 by Plaisier *et al* [Plaisier *et al.,* 2006; Rogers *et al.,* 1993]. Possibly, vascularisation in DSE is already affected by hormones, although the decidual morphology is not yet present. The decreased vessel density in DP and DB compared to DSE and DB compared to DP suggests fusion or degeneration of vascular structures. Vailhe *et al* reported a non-significant decreased vessel density in DB compared to DP, but did not study DSE [Vailhe *et al.,* 1999]. The vessel density reported in their study, ranging between 20 and 30 vessels/mm^2, is comparable with our findings. Overall, the vascular differences between the decidual tissues indicate a regulatory role in decidual vascularisation for both pregnancy-induced hormones and the EVT.

Both steroidal hormones and EVT are able to modulate vascularisation directly or indirectly via angiogenic factors like VEGF-A, PlGF, angiopoietins and hCG [Greb *et al.,* 1997; Hyder, Stancel 1999/2000; Krikun *et al.,* 2004b; Licht *et al.,* 2003; Simoncini *et al.,* 2003; Toth *et al.,* 1994/2001; Wulff *et al.,* 2000; Zygmunt *et al.,* 2002]. Therefore, we studied whether the changes in vascularisation in the decidual tissues correlated with a differential expression of angiogenic factors. First, gene and protein expressions in DSE and DP were compared. Although TIE-2 mRNA was the only differentially expressed mRNA, its protein expression was not significantly differing between DSE and DP. This suggests that TIE-2 protein expression is not influenced by pregnancy-induced hormones, which is in agreement with the finding that TIE-2 protein expression is not regulated during the menstrual cycle [Krikun *et al.,* 2000]. The presence of endothelial TIE-2 in all decidual tissues suggests that TIE-2 plays a rather general role in decidual vascularisation.

The differential expression of the studied angiogenic factors did not provide a satisfactory explanation for the vascular differences between DSE and DP. Possibly, steroidal hormones act through other angiogenic factors or exert direct effects on decidual endothelium.

Subsequently, the expressions of angiogenic factors in DB were compared to those in DSE and DP. Ang-1, Ang-2 and TIE-2 mRNA expressions were significantly lower in DB compared to both DSE and DP and therefore appear to be regulated by the EVT. The decreased TIE-2 expression might result from the decreased Ang-1 and/or Ang-2 levels, since mice lacking Ang-1 have reduced TIE-2 mRNA levels [Suri *et al.,* 1996]. At protein level, the Ang-2/Ang-1 ratio was significantly elevated in DB compared to DP and DSE, suggesting that vessel destabilization is favoured over vessel maturation in DB. The high amount of Ang-2, together with the abundant VEGF expression, may lead to more angiogenesis in DB which is in concordance with the increased vascular surface in DB [Asahara *et al.,* 1998].
Interestingly, all three decidual tissues showed Ang-2/Ang-1 ratios in favour of Ang-2. Embryonic implantation normally occurs under hypoxic conditions and oxygen levels in decidua are relatively low in the first-trimester, ranging between 50 and 75 mmHg [Jauniaux *et al.,* 2000]. Hypoxia enhances Ang-2 transcription and destabilizes Ang-1, which may result in the increased Ang-2/Ang-1 ratio [Zhang *et al.,* 2001]. Although implantation appears to favour Ang-2, a small amount of Ang-1 must be essential for implantation since disruption of the Ang-1 gene in mice results in embryonic lethality [Suri *et al.,* 1996].
Another striking observation was the abundant induction of PlGF mRNA and proteins in DB compared to DSE and DP. The increased PlGF protein expression was not only due to PlGF production by EVT, since all studied cell types, except PSMCs, showed increased PlGF protein expression in DB. Furthermore, uterine NK cells are known for their PlGF production as well and probably contribute to the production of angiogenic factors in decidua as well.
Interestingly, PlGF and its receptor, Flt-1, were mainly expressed on endothelium of DB. PlGF, together with Flt-1, is able to potently induce angiogenesis resulting in mature and stable vessels [Arroyo *et al.,* 2004; Carmeliet *et al.,* 2001; Khaliq *et al.,* 1996; Luttun *et al.,* 2002; Torry *et al.,* 1999]. The induction of PlGF and Flt-1 mRNAs and proteins in decidua basalis suggests that both factors play a specific role in regulating angiogenesis at the site of implantation and may, at least partially, account for the vascular changes noticed in decidua basalis.
VEGF mRNAs and proteins were abundantly, but not differentially, expressed in all decidual tissues and KDR, Flt-1 and VEGF proteins were expressed on endothelium in

DB and DP. These findings suggest a general role for VEGF in angiogenesis in human decidual tissues.

As gestation progresses, vascular adaptation continues which manifested itself in the present study by decreased vessel density in DB and DP. VEGF and Ang-2 mRNA and VEGF, Ang-1 and -2 protein expressions were increased in late first-trimester DB. These increased levels of the angiogenic factors suggest increased angiogenic activity at the implantation site as gestation progresses. This was further supported by the increased endothelial expression of PlGF and VEGF proteins in late first-trimester decidua.

The vascular adaptation to implantation is clearly important in the development of a healthy pregnancy. We have shown that vascular adaptation occurs under influence of both pregnancy-induced hormones and the extra-villous trophoblast, by enhancing the vascular surface, reducing vessel density and regulating the expression of angiogenic factors. VEGF-A, via KDR, probably plays a general role in supporting decidual vascularisation, whereas the angiopoietins, via TIE-2, and PlGF, via Flt-1, appear to be key regulators of the vascular adaptive process and may, partially, account for the vascular changes at the site of embryonic implantation in decidua basalis. Finally, studying vascularisation during mammalian implantation hopefully leads to new understandings of currently inexplicable events associated with pregnancy, such as pre-eclampsia and missed abortions.