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

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Chapter 8

The expression of angiogenic factors in first- trimester human decidua of pregnancies further complicated by pre-eclampsia or foetal growth restriction.

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ABSTRACT

Objective: Disturbances in decidual and placental vascular development may play a role in the pathogenesis of pregnancy complications. This study focused on the role of angiogenic factors in the first-trimester in the pathogenesis of pre-eclampsia (PE) and/or foetal growth restriction (FGR).

Materials and Methods: First-trimester decidua was obtained during chorionic villous sampling. The expression of VEGF-A, PlGF, Flt-1, KDR, Angiopoietin-1, Angiopoietin-2 and TIE-2 mRNA was determined by RT-PCR and related to the pregnancy outcome.

Results: First-trimester decidua expressed all angiogenic factors. VEGF-A, PlGF, KDR, Ang-1, Ang-2 and TIE-2 appeared increased in FGR compared to matched controls, whereas PlGF, Ang-1 and TIE-2 appeared increased in PE compared to controls. Flt-1 mRNA levels were comparable in both PE and FGR compared to the controls. The differential expression of angiogenic factors appeared to be more prominent in FGR than in PE.

Conclusions: These first-trimester samples provided a unique opportunity to obtain information regarding the onset of PE and FGR. First-trimester changes in angiogenic factor expression may well occur as a compensatory mechanism. In turn, this may unintentionally set the stage for increased angiogenesis and altered decidual/placental vascular adaptation, which may be part of the pathogenesis of PE and/or FGR.

INTRODUCTION

Disturbances in decidual and placental vascular development may play a role in complications of pregnancy, like pre-eclampsia (PE) and foetal growth restriction (FGR) [Ahmed, Perkins 2000; Ong *et al.,* 2000; Torry *et al.,* 2004]. PE affects 5-10% of pregnancies and is responsible for substantial maternal and neonatal morbidity and mortality. Predominant features are maternal systemic vascular endothelial dysfunction and microangiopathy. Target organs are the brain (seizures or eclampsia), liver (Hemolysis, Elevated Liver enzymes, Low Platelets (HELLP) Syndrome), kidney (proteinuria) and the utero-placental unit (fetal distress and growth restriction). Failure of placental vascular development has been linked to the pathogenesis of PE and FGR [Luttun, Carmeliet 2003; Maynard *et al.,* 2003].

Decidual vascular adaptation to pregnancy is characterised by high levels of both angiogenesis, the formation of new vessels from existing vasculature, and vasculogenesis, blood vessel generation from angioblast precursor cells [Burton *et al.,* 1999; Pijnenborg *et al.,* 1983]. Additionally, vascular mimicry or pseudo-vasculogenesis is also taking place where cytotrophoblasts convert to an endothelial phenotype. Failure of angiogenesis and pseudo-vasculogenesis has been proposed as processes in the inception of PE and FGR [Zhou *et al.,* 2002].
Numerous factors are thought to play a role in normal vascular adaptation to implantation [Geva *et al.,* 2002]. Vascular Endothelial Growth Factor-A (VEGF-A) and Placental Growth Factor (PlGF), are probably the best studied factors [Das *et al.,* 1997a; Plaisier *et al.,* 2007; Chung *et al.,* 2004]. VEGF interacts with 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-A have synergistic effects regarding angiogenesis, but vessels induced by PlGF are more mature and stable than vessels induced by VEGF-A [Carmeliet *et al.,* 2001; Luttun *et al.,* 2002]. PlGF is abundantly expressed in human placenta. Both VEGF-A and PlGF may be important paracrine regulators of decidual angiogenesis and autocrine mediators of trophoblast function [Plaisier *et al.,* 2007; Sherer, Abulafia 2001]. The soluble variant of Flt-1 has been associated with the pathogenesis of PE [Luttun, Carmeliet 2003; Maynard *et al.,* 2003].
A second family of growth factors, the angiopoietins, is also known for their regulating capacities regarding angiogenesis [Geva, Jaffe 2000]. Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) bind with equal affinity to their receptor TIE-2, but have different functions. Ang-1 maintains vessel integrity and plays a role in the later stages of vascular remodelling [Geva, Jaffe 2000]. Ang-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 and Ang-2 have both been detected in decidual and placental tissues [Maisonpierre *et al.,* 1997; Plaisier *et al.,* 2007].
Various decidual celltypes are capable of producing angiogenic factors. We recently showed the production of PlGF, KDR, Flt-1, Ang-2 and TIE-2 by endothelial cells and extra-villous trophoblasts. Decidual stromal cells, glandular epithelium and perivascular smooth muscle cells were found to produce all studied angiogenic factors [Plaisier et al,. 2007]. Uterine NK cells are also abundantly present in first-trimester decidua and are known to produce PlGF, VEGF, Ang-1 and Ang-2 [Li *et al.,* 2001a].

The possible correlation of PE and FGR with impaired vascular development has been studied in placental tissues mainly after the onset of disease [Geva *et al.,* 2002; Roberts, Cooper 2001; Taylor *et al.,* 2003]. However, whether decidual vascularisation is also related to the pathogenesis of both diseases is not known. Information regarding the expression of angiogenic factors in first-trimester tissues would be helpful in understanding PE and FGR. The present study focused on the role of first-trimester angiogenic growth factors in the pathogenesis of PE and/or FGR. The expression of VEGF-A, PlGF, Flt-1, KDR, Ang-1, Ang-2 and TIE-2 was determined in first-trimester decidua obtained during routine chorion villous sampling. The included patients were followed throughout their pregnancy to be able to relate the angiogenic factor expression to the pregnancy outcome, i.e. uncomplicated, PE or FGR.

MATERIALS & METHODS

Study group and tissue collection

Decidual samples were obtained from viable first-trimester pregnancies during routine chorionic villous sampling (CVS). CVS was performed vaginally between 10 and 12 weeks of gestation. Main indications were maternal age and serum screening related risk for aneuploidy. Gestational age was calculated according to the last menstrual period and confirmed by ultrasound (crown lump length). After CVS, surplus decidual tissue was microscopically separated from the chorion villi and immediately stored at -20 °C until RNA isolation. One sample per person was collected with an average size of 5-15 mg.
Follow-up of the pregnancies was available through a postpartum questionnaire. Patients experiencing FGR (FGR, n=10) and PE/HELLP (n=9) were selected. PE was defined as diastolic blood pressure (BP) of minimal 90 mmHg, or a raise of 15 mmHg compared to first-trimester BP, combined with proteinuria of at least 300 mg/day. For FGR, criteria

according to Kloosterman were used [Kloosterman 1969]. Birth weight was corrected for gestational age and FGR was defined as a corrected birth weight below the 5th percentile. All cases were matched to controls based on gestational age, maternal age and parity at time of CVS (n=19). Patients with concurrent morbidity (e.g. diabetes) or medication were excluded. Foetal karyotyping showed no chromosomal abnormalities. The weight and the number of smokers were comparable between cases and controls. Patients were informed that surplus material was used for research, according to the "Guideline for Good Use" by the Federation of Medical Scientific Associations and informed consent was provided by all study subjects. Patient characteristics are described in Table 1.

RNA isolation and cDNA synthesis

Tissue samples were incubated in 1.5 ml RNA Later Ice (Ambion, Austin, TX, USA) for 48 hours at -20°C. After centrifugation, the tissue samples were lysed in lysisbuffer (RLT buffer (Qiagen) and β-mercapto-ethanol) using plastic mortars and RNA isolation was performed using RNAeasy columns (Qiagen Benelux BV, Venlo, Netherlands) following recommended protocol. mRNA was dissolved in RNAse free water. mRNA 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 (Ready-to-go You-Prime first strand beads, Amersham Biosiences, Buckinghamshire, UK). The obtained 32 μl cDNA was diluted 1:3.

Real-time RT-PCR

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, Flt-1, 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 (all purchased from Applied Biosystems, Foster City, CA, USA).

Table 1. Characteristics of the FGR and PE study groups and matched control groups.

		FGR (n=10) mean ± SD	Control (n=10) mean ± SD	PE/HELLP (n=9) mean ± SD	Control (n=9) mean ± SD
At CVS	**Age (years)**	39.5 ± 2.1	39.6 ± 2.1	39.2 ± 1.9	39.2 ± 1.9
	GA (days)	75.6 ± 3.2	76.5 ± 2.5	78.6 ± 3.9	77.3 ± 4.4
	Parity	1.5 ± 1.4	1.1 ± 0.6	0.7 ± 0.9	0.7 ± 0.9
At delivery	**GA (days)**	278 ± 11	282 ± 12	261 ± 22	271 ± 10
	Birth weight (g)	2477 ± 286*	3648 ± 475	2648 ± 798	3420 ± 382

* $p<0.05$ versus control group

β-actin, β_2-microglobulin, and cyclophilin were also used as reference genes and showed similar results (data not shown).
RT-PCR for target gene/GAPDH pairs was 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 and mRNA levels were expressed in cycle threshold (Ct). To correct for the RNA input, the difference in Ct values of target and reference genes (dCt = Ct(target gene) – Ct(GAPDH)) was calculated. The difference between cases and controls (ddCt) was calculated as ddCt= dCt(case) – dCt(control) and the fold induction was calculated as 2^{-ddCt}. In addition, Ct values were quantified into ng/μl using a standard curve of total RNA. Cases and controls were compared by the ratio ng/μl(case)/ ng/μl(control). Water and negative-RT samples, obtained by the omission of the reverse transcriptase enzyme in the cDNA reaction, were used as negative controls.

Statistics

The mRNA expression levels were compared between cases and matched controls. A general linear model for repeated measurements, ANOVA, was performed to analyse the paired data between matched cases and controls (SPSS 11.5). P-values of < 0.05 were considered significant.

RESULTS

The expression of the angiopoietins and their receptor TIE-2 and of VEGF, PlGF and their receptors KDR and Flt-1 were evaluated. The mRNA levels in all cases were calculated and expressed as ddCt, ng/μl and as fold induction compared to the control group (Table 2 and 3). All factors were present at mRNA level in decidua of cases as well as controls.

Control group

The control patients were matched to the cases; ten controls to FGR patients and nine controls to PE patients. First, mRNA expression was analysed in all the control patients. In these controls, PlGF showed the most abundant expression with a mean of 4.6 ng/μl (± 1.8). KDR, Flt-1 and Ang-2 showed a moderate expression with means of 0.4 ng/μl (± 0.1), 0.5 ng/μl (± 0.1) and 0.8 ng/μl (± 0.3) respectively. VEGF-A, Ang-1 and TIE-2 were only dimly expressed with means of 0.04 ng/μl (± 0.01), 0.01 ng/μl (± 0.00) and 0.15 ng/μl (± 0.06) (data not shown).

Table 2. First-trimester decidual mRNA expression of angiogenic factors (in cycle threshold (Ct)).

	mRNA content (dCt) mean ± SEM [a]		Fold induction mean ± SEM [b]	
Angiogenic Factor	**FGR (n=10)**	**PE (n=9)**	**FGR (n=10)**	**PE (n=9)**
VEGF-A	-1.5 ± 1.2	-0.5 ± 0.8	83 ± 70	3 ± 1
PlGF	0.5 ± 1.6	-2.0 ± 1.4	31 ± 21	51 ± 34
KDR	-0.5 ± 1.8	-1.8 ± 1.1	32 ± 25	17 ± 9
Flt-1	0.4 ± 0.6	-1.1 ± 0.7	2 ± 0.9	8 ± 6
Ang-1	-1.5 ± 1.9	-1.7 ± 1.6	84 ± 50	68 ± 40
Ang-2	-0.4 ± 1.3	0.1 ± 1.3	11 ± 5	6 ± 5
TIE-2	-1.2 ± 1.5	-3.1 ± 1.3	34 ± 25	110 ± 83

First-trimester decidual mRNA expression was determined by RT-PCR and compared between pregnancies resulting in FGR or PE and their matched controls. The 'repeated measures ANOVA' was performed to analyse the data.

[a] mRNA content was expressed in cycle threshold (Ct). Mean Ct per gene: GAPDH 25, VEGF-A 26.0, PlGF 29.2, KDR 28.5, Flt-1 28.4, Ang-1 33, Ang-2 27.4, and TIE-2 32.1. The Ct values were corrected for the mRNA input, by calculating the difference in Ct of target and reference genes: dCt = Ct(target gene) – Ct(GAPDH).

[b] The difference between cases and controls (ddCt) was calculated as ddCt= dCt(case) – dCt(control). Fold induction in cases compared to controls was calculated as 2^{-ddCt}.

Fetal growth restriction group

The FGR group showed no significant differential expression compared to the control group (100%). Strikingly, the expression of VEGF-A (53-fold), PlGF (33-fold), KDR (26-fold), Ang-1 (59-fold), Ang-2 (8-fold) and TIE-2 (37-fold) appeared unregulated in cases compared to controls. However, significance was not reached due to the large inter-individual variation. Flt-1 showed comparable expression levels in cases and controls. The same pattern was found with regard to the data described as dCt (Table 2 and 3).

Pre-eclampsia group

The PE group showed no significant differential expression compared to the control group (100%). PlGF (35-fold), Ang-1 (29-fold) and TIE-2 (68-fold) appeared unregulated in cases compared to controls. However, these differences were not significant due to the large inter-individual variation. VEGF-A, KDR, Flt-1 and Ang-2 showed comparable expression levels in cases and controls. With regard to the data described as dCt, the same pattern was found (Table 2 and 3).

Table 3. First-trimester decidual mRNA expression of angiogenic factors (in ng/ μl).				
	mRNA content mean ± SEM [a]		**Fold induction mean ± SEM** [b]	
Angiogenic Factor	**FGR (n=10)**	**PE (n=9)**	**FGR (n=10)**	**PE (n=9)**
VEGF-A	0.1 ± 0.0	0.1 ± 0.0	53 ± 46	2 ± 1
PlGF	4.1 ± 1.8	8.2 ± 3.1	33 ± 21	35 ± 24
KDR	0.7 ± 0.3	1.7 ± 0.8	26 ± 17	13 ± 8
Flt-1	0.6 ± 0.2	2.0 ± 1.3	2 ± 1	3 ± 2
Ang-1	0.02 ± 0.0	0.4 ± 0.4	59 ± 34	29 ± 21
Ang-2	0.4 ± 0.2	3.6 ± 2.0	8 ± 4	5 ± 3
TIE-2	0.9 ± 0.8	19.6 ± 19.5	37 ± 30	68 ± 47

First-trimester decidual mRNA expression was determined by RT-PCR and compared between pregnancies resulting in FGR or PE and their matched controls. The 'repeated measures ANOVA' was performed to analyse the data.
[a] mRNA content was expressed in cycle threshold (Ct). Mean Ct per gene: GAPDH 25, VEGF-A 26.0, PlGF 29.2, KDR 28.5, Flt-1 28.4, Ang-1 33, Ang-2 27.4, and TIE-2 32.1. Cycle threshold were converted into ng/μl using a standard curve of total RNA. Values were corrected for mRNA input by calculating the ratio (ng/μl (target gene))/ (ng/μl (GAPDH)) and these values are expressed as "mRNA content" in the table.
[b] Fold induction in cases compared to controls was calculated as the ratio ng/μl (case)/ ng/μl (controls).

DISCUSSION

The results showed that first-trimester decidual tissues obtained from chorionic villous sampling expressed all examined angiogenic factors. mRNA levels of VEGF-A, PlGF, KDR, Ang-1, Ang-2 and TIE-2 appeared unregulated in FGR cases compared to matched controls. In addition, PlGF, Ang-1 and TIE-2 mRNA appeared unregulated in PE cases compared to matched controls. However, the large inter-individual variation disallowed an unequivocal conclusion. The differential expression of angiogenic factors appeared more prominent in FGR than in PE.

The origin of both PE and FGR probably starts in the first-trimester, before the onset of overt disease during the last trimester. Our first-trimester decidual samples provide a unique opportunity to obtain information regarding the onset of PE and FGR. The fact that the decidual samples were obtained together with villous tissue offers valid information on the original localization *in utero*, namely underneath the implantation site. Shortcomings are also present. No histological investigation could be performed on the number of trophoblasts and other cell types, because of the small amounts of material in each sample. Also, the study groups are small because of the rarity of the samples. This results in rather high standard deviations, which makes interpretation of the data

difficult. However, the 10- to 100-fold induction of genes in these small groups promises significant differences when groups could be expanded.

Current knowledge regarding PE suggests that defective vascular remodelling of maternal spiral arteries leads to impaired invasion of trophoblasts, placental insufficiency and ischemia [Luttun, Carmeliet 2003]. The affected placenta releases soluble anti-angiogenic factors, like soluble Flt-1 (sFlt-1) and endoglin (sEng) [Maynard *et al.,* 2003; Clark *et al.,* 1998b; Tjoa *et al.,* 2007]. These factors alter the angiogenic balance, mainly by neutralising VEGF and PlGF. Furthermore, these factors are able to induce systemic endothelial dysfunction and finally to induce clinical PE [Maynard *et al.,* 2003; Ahmad, Ahmed 2004; Hayman *et al.,* 1999; Sugimoto *et al.,* 2003]. The serum and placental levels of these factors have been studied extensively in term pregnancies, without reaching complete consensus with regard to VEGF. Several reports described increased, comparable and even decreased VEGF levels in placenta of term PE pregnancies [Geva *et al.,* 2002; Chung *et al.,* 2004; Ranheim *et al.,* 2001; Sgambati et al 2004]. PlGF was decreased in serum of PE in the third-trimester [Lam *et al.,* 2005; Roberts, Cooper 2001]. Term placental tissue showed reduced Ang-2 mRNA and equal Ang-1 mRNA in PE [Zhang *et al.,* 2001]. No reports on these angiogenic factors are available regarding first-trimester tissues.
In the present study, mRNA levels of PlGF, Ang-1 and TIE-2 appeared unregulated in future PE patients compared to controls. VEGF-A, KDR, Flt-1 and Ang-2 showed comparable expression levels. No previous reports were found to reference these data in first-trimester tissues. Only first-trimester serum data are available, showing reduced PlGF and increased VEGF and sflt-1 in first-trimester serum of PE patients [Levine *et al.,* 2004; Bosio *et al.,* 2001].
The studied Flt-1 represents both the soluble and membrane-bound Flt-1 and unfortunately no differentiation can be made between both types. The comparable overall Flt-1 expression in PE cases probably corresponds to the described decline of membrane bound Flt-1 and the known increase of soluble Flt-1 [Tsatsaris *et al.,* 2003].

FGR has been proposed to be induced by relatively high oxygen levels, which cause a disturbed expression of angiogenic factors and altered angiogenesis in placental villi [Burton 1997; Kingdom, Kaufmann 1997; Regnault *et al.,* 2003]. These relative hyperoxic conditions may cause the decreased VEGF and increased PlGF expression in FGR placentas, since these factors are inversely regulated by oxygen [Lyall 1997; Khaliq *et al.,* 1999]. An ovine model for FGR demonstrated increased Ang-1, Ang-2 and TIE-2 levels in early gestation before FGR was clinically detected. Later in gestation decreased TIE2 expression was associated with FGR [Erickson Hagen *et al.,* 2005]. Whether decidual angiogenesis is also affected by the altered oxygen levels in FGR is still not demonstrated but plausible.

Present study showed that the mRNA levels of angiogenic factors in first-trimester decidua were altered in the FGR group compared to the control group, although differences were not significant. The expression of VEGF-A, PlGF, KDR, Ang-1, Ang-2 and TIE-2 appeared unregulated in FGR cases and Flt-1 showed comparable expression levels in cases and controls.
The relative hyperoxia in FGR and the oxygen effect on angiogenic factors are mainly described in feto-placental tissues and trophoblasts. If the theory is valid for first-trimester decidua, one would expect decreased VEGF and Ang-2 and increased Ang-1 and PlGF mRNA levels in FGR patients [Zhang *et al.,* 2001; Regnault *et al.,* 2003; Khaliq *et al.,* 1999; Sharkey *et al.,* 2000]. However, mRNA levels of all four genes appeared increased in FGR compared to matched controls. This might be explained by a different expression and regulation of angiogenic factors in first-trimester compared to third-trimester tissues. For example, VEGF-A and Ang-1 were shown to increase and Ang-2 to decrease as gestation progresses [Geva *et al.,* 2002]. In contrast, others demonstrated a decline in VEGF-A mRNA as gestation progresses [Cooper *et al.,* 1996]. In addition, oxygen influences in decidua may differ from its influences in villous tissue and/or trophoblasts. No references were found regarding oxygen regulation in decidua. Finally, histological differences or differences in the number of trophoblasts between the studied specimens cannot be ruled out.

The differential expression of angiogenic factors appeared more prominent in FGR than in PE and therefore a more pronounced disbalance between angiogenic factors might be involved in the pathogenesis of FGR. Overall, enhanced angiogenic factors in both patient groups might represent an (ineffective) vascular rescue mechanism in response to unidentified situation(s) and/or event(s). This compensatory mechanism may in turn unintentionally induce increased non-branching angiogenesis, altered decidual and placental vascular adaptation and functional placental insufficiency that may all result in PE and/or FGR during late gestation.