Placental expression of VEGF family mRNA in adverse pregnancy outcomes

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Abstract
Introduction: The pregnancy complications preeclampsia, gestational hypertension, small for gestational age infants (SGA) and pre-term birth (PTB) affect approximately 21% of all pregnancies. The Vascular Endothelial Growth Factor family (VEGF) is implicated in the pathogenesis of these complications. We aimed to evaluate the placental mRNA expression of VEGFA, PGF, FLT1 and KDR in pregnancies complicated by preeclampsia, gestational hypertension, SGA infants and pre-term birth.

Method: Placentae were collected at delivery from women with pregnancies complicated by preeclampsia (n = 18), gestational hypertension (n = 15), normotensive SGA infants (n = 13), late spontaneous pre-term birth (n = 10) and uncomplicated pregnancy (n = 30). RNA was extracted and VEGFA, PGF, FLT1 and KDR expression were quantified using qRT-PCR. Kruskal Wallis test was used to compare placental mRNA expression in the adverse pregnancy outcome groups compared to uncomplicated term pregnancy.

Results: Compared to placental mRNA from uncomplicated pregnancies, VEGFA (p = 0.006), PGF (p < 0.001), KDR (p < 0.001) and FLT1 (p = 0.02) mRNA were reduced in preeclamptic placentae; VEGFA (p < 0.001), PGF (p = 0.01) and KDR (p = 0.008) mRNA were reduced in placentae from pregnancies complicated by gestational hypertension; VEGFA (p = 0.03) mRNA was reduced in normotensive SGA pregnancies; VEGFA (p = 0.008), PGF (p = 0.01), KDR (p = 0.04) and FLT1 (p = 0.02) mRNA were reduced in placentae from late PTB.

Conclusion: VEGF family of angiogenic growth factor mRNA expression in the placenta is reduced in gestational hypertensive disorders, SGA and in pre-term birth.

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1. Introduction

The pregnancy complications preeclampsia, gestational hypertension, small for gestational age infants (SGA) and spontaneous pre-term birth affect approximately 21% of all pregnancies [12]. Early placentation defects, including impaired maternal spiral artery remodelling and placental villous vascularisation have been demonstrated in pregnancies complicated by preeclampsia, SGA infants and pre-term birth [3–6]. Many molecular pathways are involved in the pathogenesis of these placentation defects, of which the vascular endothelial growth factor (VEGF) family mediated angiogenic pathway is recognized as playing an important role [7].

The VEGF family consists of VEGFA, VEGFB, VEGFC, VEGFD, placental growth factor (PGF) and their receptors VEGFR1 (FLT1, fms-like-tyrosine-kinase receptor 1), VEGFR2 (KDR, kinase insert domain receptor: FLK in mice), VEGFR3 and the co-receptors Neuropilin-1 and Neuropilin-2. Of these, VEGFA and PGF acting through KDR and FLT1 are known to regulate early placental vascular development.

Gene ablation studies in mice demonstrate that homozygous gene mutations in VEGFA, FLT1 and FLK result in early embryonic death demonstrating that these molecules are critical for embryonic angiogenesis [8–12]. During pregnancy, VEGFA, PGF, FLT1 and KDR are expressed in villous and extravillous trophoblasts, villous vascular endothelium, as well as in decidual natural killer cells, and the level of expression is known to be altered in adverse pregnancy outcomes [13–18].

To date, most studies on placental expression of the VEGF family of angiogenic growth factors in adverse pregnancy outcomes have focused mainly on the expression of VEGFA in preeclampsia. Some studies have shown that placental expression of VEGF mRNA at term is reduced [19,20] in preeclamptic placentae compared to normal placentae while others have demonstrated an increase [21] or no difference [22,23]. The aim of this study was to evaluate the
expression of VEGFA, PGF, FLT1 and KDR mRNA in pregnancies complicated by preeclampsia, gestational hypertension, SGA infants or pre-term birth.

2. Materials and methods

This is a nested case control study where participants were recruited from the Screening for Pregnancy Endpoints (SCOPE) study. The SCOPE study is an international, multicentric, prospective cohort study with the aim of developing screening tests to predict preeclampsia, SGA infants and pre-term birth across different populations [24]. The participants were recruited to the Adelaide cohort of the SCOPE study and ethics approval was gained from the local ethics committees. All women provided written informed consent for collection of placentae at delivery.

Nulliparous women with singleton pregnancies were recruited between September 2005 and September 2008. Women considered at high risk for preeclampsia, SGA infants or pre-term birth were not eligible to participate [25].

Maternal data collected included demographic information, medical history, previous obstetric history, family history of obstetric complications and medical disorders. Current pregnancy data included information on any complications during current pregnancy, diet, smoking, alcohol and the use of recreational drugs [24]. Maternal physical measurements obtained at 15 weeks of gestation included height, weight and blood pressure.

Gestational hypertension was defined as systolic blood pressure $\geq$ 140 mmHg or diastolic blood pressure $\geq$ 90 mmHg, or both, on at least two occasions 4 h apart after 20 weeks’ gestation. Preeclampsia was defined as gestational hypertension with significant proteinuria and/or any multisystem complication of the disease [26]. Severe hypertension was defined as systolic blood pressure $\geq$ 170 mmHg or diastolic blood pressure $\geq$ 110 mmHg. Small for gestational age was defined as a birth weight $<$ 10th customised centile adjusted for maternal height, weight, parity and ethnicity, as well as gestational age at delivery and infant sex [27]. Normotensive SGA was defined as birth of an SGA infant where the mother did not have hypertension. Spontaneous pre-term birth was defined as spontaneous pre-term labour or pre-term premature rupture of membranes resulting in pre-term birth at $<$ 37 weeks. Uncomplicated pregnancy was defined as a pregnancy with no antenatal medical or obstetric complications and resulting in the delivery of an appropriately grown, healthy infant at $\geq$ 37 weeks of gestation.

3. Placental tissue

The study groups considered were: (1) preeclampsia ($n$ = 18); (2) gestational hypertension ($n$ = 15); (3) normotensive SGA ($n$ = 13); (4) spontaneous pre-term birth ($n$ = 10) and (5) uncomplicated pregnancy ($n$ = 30). The placental samples were randomly collected from a small subset of SCOPE women by labour ward midwives usually within 30 min of delivery. As far as we are aware there was no selection bias although if the baby or woman were very sick their placentae would not have been sampled. The placentae were weighed and a 1 cm$^2$ full thickness block of tissue was immersed in RNAlater (Ambion, Austin, Texas) for at least 24 h and frozen at $-80 ^\circ$C for subsequent RNA extraction. Total RNA was isolated from 100 mg of each placental villous tissue using the TRIzol method according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Potential RNA degrading contaminants were removed by treatment with the DNA-free™ DNase Treatment Kit (Ambion, Austin, Texas) and RNA integrity was determined by gel electrophoresis.

4. Quantitative RT-PCR

For each placental sample, 2 $\mu$L of RNA was reverse transcribed to cDNA using random hexamer primers (GeneWorks, Adelaide, SA, Australia), and Superscript III (Invitrogen, Carlsbad, CA, USA) according to the manufacturers’ instructions. Quantitative RT-PCR was performed on a Rotor-Gene™ 6000 real-time PCR machine (Corbett Research, Sydney, Australia). All reactions were set up using a CFX96™ liquid handling system (Bio-Rad, Hercules, CA, USA). The endogenous control, 18S RNA was used for normalization of the raw data. The primer sequences for VEGFA, PGF, FLT1, KDR and 18S rRNA are detailed in Table 1. All reactions were carried out in 10 $\mu$L of mixture containing, 5 $\mu$L of SYBR Green PCR Master Mix (2X) (Applied Biosystems, Warrington, UK), 0.5 $\mu$L each of forward and reverse primer, 2 $\mu$L cDNA and 2 $\mu$L of sterile water for injection. The thermal cycling conditions were 10min at 95 $^\circ$C, then with 40 cycles at 95 $^\circ$C for 15 s, 60 $^\circ$C for 10 s and 72 $^\circ$C for 10 s. All samples were assayed in triplicate and a six point standard and an internal control were assayed in triplicate on each plate. Relative mRNA expression was determined by the Standard Curve method [28].

5. Statistics

Chi square test was used to compare categorical variables and ANOVA was used to compare continuous variables that were normally distributed. Placental expression of VEGF family angiogenic growth factors in complicated term pregnancy was compared to the expression in uncomplicated term pregnancy using Kruskal Wallis test since these data were not normally distributed. All data analyses were performed using PASW version 17.02 (SPSS, Chicago, IL). Results are reported as N (%) or median and range. P $<$ 0.05 was considered statistically significant.

6. Results

Characteristics of the study population are detailed in Table 2 and Supplementary Table 1 [29]. In the study cohort, 50 (58.1%) women had unassisted vaginal delivery, 17 (19.8%) had operative vaginal delivery, 17 (19.8%) underwent Caesarean section after the onset of labour and 2 (2.3%) underwent elective Caesarean section. Of the preeclamptic women, 9 (50%) had severe hypertension and delivered SGA infants. Four women in the preeclampsia group and one woman in the normotensive SGA group delivered prior to 37 weeks gestation. There was no significant difference in placental mRNA expression of VEGFA, PGF, KDR and FLT1 based on the mode of delivery (data not presented). Placental mRNA levels were unaffected by smoking or alcohol intake at 15 weeks gestation (data not presented). Placental weight was not related to mRNA expression of any gene (data not presented).

7. Placental VEGF family mRNA expression in preeclampsia

Placental expression of VEGFA, PGF, KDR and FLT1 mRNA were reduced by 53% ($p = 0.006$), 60% ($p < 0.001$), 55% ($p < 0.001$) and by 45% ($p = 0.02$) respectively, in preeclamptic placentae compared to those from uncomplicated pregnancy (Fig. 1). The results were unchanged when placentae from the women who delivered pre-term and those who delivered SGA infants were excluded from the analyses.
Table 2
Characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control n = 30</th>
<th>Preeclampsia n = 18</th>
<th>P</th>
<th>GH n = 15</th>
<th>P</th>
<th>NSGA n = 13</th>
<th>P</th>
<th>PTB n = 10</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>23.6 ± 4.8</td>
<td>24.1 ± 4.7</td>
<td>0.7</td>
<td>23.9 ± 4.5</td>
<td>0.8</td>
<td>23.7 ± 4.8</td>
<td>0.9</td>
<td>21.6 ± 4.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Maternal BMI (kg/m²)</td>
<td>27.4 ± 5.1</td>
<td>30.1 ± 7.1</td>
<td>0.2</td>
<td>28.4 ± 5.1</td>
<td>0.5</td>
<td>28.1 ± 5.9</td>
<td>0.7</td>
<td>24.8 ± 6.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Neonatal birthweight (g)</td>
<td>3547 ± 370</td>
<td>3212 ± 660</td>
<td>0.04</td>
<td>3466 ± 381</td>
<td>0.4</td>
<td>2844 ± 265</td>
<td>&lt;0.001</td>
<td>3119 ± 430</td>
<td>0.2</td>
</tr>
<tr>
<td>Customised birthweight centile</td>
<td>53 ± 23</td>
<td>32 ± 31</td>
<td>0.05</td>
<td>44 ± 24</td>
<td>0.2</td>
<td>5.0 ± 3.0</td>
<td>&lt;0.001</td>
<td>44 ± 38</td>
<td>0.6</td>
</tr>
<tr>
<td>Gestational age at delivery (weeks)</td>
<td>40.1 (38–41)</td>
<td>38.0 (32–41)</td>
<td>&lt;0.001</td>
<td>39.3 (37–41)</td>
<td>0.03</td>
<td>39.4 (36–41)</td>
<td>&lt;0.001</td>
<td>35.4 (34–36)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Placental weight</td>
<td>621 ± 125</td>
<td>570 ± 148</td>
<td>0.9</td>
<td>523 ± 79</td>
<td>0.07</td>
<td>478 ± 94</td>
<td>0.005</td>
<td>474 ± 93</td>
<td>0.6</td>
</tr>
<tr>
<td>Birthweight: Placental weight ratio</td>
<td>6.0 ± 0.9</td>
<td>5.5 ± 1.1</td>
<td>0.1</td>
<td>6.7 ± 0.7</td>
<td>0.06</td>
<td>5.8 ± 0.9</td>
<td>0.4</td>
<td>5.6 ± 0.8</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Data are either N (%) or Mean ± SD, comparisons are with uncomplicated term pregnancy using ANOVA.
Bold values indicates significant p values.
* Adjusted for gestational age at delivery.
* Mean (range).

8. Placental VEGF family mRNA expression in pregnancies complicated by gestational hypertension

Placental expression of VEGFA, PGF and KDR mRNA were reduced by 47% (p < 0.001), 27% (p = 0.01), and by 39% (p = 0.008) respectively, in pregnancies complicated by gestational hypertension compared to uncomplicated pregnancy (Fig. 1). FLT1 mRNA expression was similar between the two groups (p = 0.4).

9. Placental VEGF family mRNA expression in normotensive SGA pregnancies

Placental expression of VEGFA mRNA was reduced by 42% (p = 0.03) in normotensive SGA compared to uncomplicated pregnancy (Fig. 1). Significant differences were not observed in the expression of PGF (p = 0.08), KDR (p = 0.5) and FLT1 (p = 0.5) mRNA between the two groups. The results were unchanged when the placenta from the woman who delivered pre-term was excluded from the analyses.

10. Placental VEGF family mRNA expression in spontaneous pre-term birth

Placental expression of VEGFA, PGF, KDR and FLT1 mRNA were reduced by 58% (p = 0.008), 40% (p = 0.01), 29% (p = 0.04) and by 53% (p = 0.02) respectively, in spontaneous pre-term birth compared to those from uncomplicated pregnancy (Fig. 1).

Fig. 1. Placental expression of VEGF family mRNA in adverse pregnancy outcomes compared to uncomplicated term pregnancy. Data are presented as median (range) and are analysed using Kruskal Wallis test; PE, preeclampsia; GH, gestational hypertension; NSGA, normotensive SGA, PTB, pre-term birth; (A) VEGFA mRNA expression *p < 0.006, **p < 0.001, ***p < 0.003, ****p < 0.005; (B) PGF mRNA expression *p < 0.001, **p < 0.01, ***p < 0.08, ****p < 0.01; (C) KDR mRNA expression *p < 0.001, **p < 0.008, ***p < 0.5, ****p < 0.04; (D) FLT1 mRNA expression *p = 0.03, **p = 0.4, ***p = 0.5, ****p = 0.02.
11. Discussion

This is the first study to report placental expression of VEGFA, PGF, KDR and FLT1 genes in several pregnancy complications within one cohort. The overall results of our study demonstrate that placental mRNA expression of the VEGF family of angiogenic growth factors is reduced in pregnancies complicated by preeclampsia, gestational hypertension, SGA infants and spontaneous pre-term birth compared to uncomplicated term pregnancy. Placental expression of VEGFA mRNA has been localized to villous and extravillous trophoblasts, villous mesenchyme, Hofbauer cells and maternal decidual cells [13–17] throughout gestation. In the first trimester, PGF is mainly expressed in extravillous trophoblasts within the maternal decidua but towards term the expression is abundant in villous trophoblasts [17,18,30]. The expression pattern of FLT1 is similar to that of VEGFA while abundant KDR expression is localized to vascular endothelial cells [13,31].

VEGFA exerts its effects via both FLT1 and KDR while PGF acts only through FLT1 [32]. VEGFA acting through FLT1 and KDR is known to regulate early placental villous vasculogenesis and branching angiogenesis which continues up to 25 weeks of gestation [33,34]. Then onwards, angiogenesis switches from branching angiogenesis to non-branching which continues to term and is known to be regulated by PGF acting through FLT1 [7]. Placentae from pregnancies complicated by growth restriction fetuses, as well as those complicated by preeclampsia plus growth restriction are characterised by a reduction in the number of chorionic villi and their accompanying vasculature [4,35].

In addition to its role in villous angiogenesis, the VEGF family is known to regulate maternal spiral artery remodelling [36]. Early pregnancy is associated with an influx of leukocytes into the decidua including uterine natural killer cells (uNK) and macrophages. Uterine NK cells isolated from first trimester decidua secrete many angiogenic growth factors including VEGFA and PGF and un-remodelled spiral arteries express KDR [37]. Uterine NK cells are a major source of angiogenic growth factors at the maternal–fetal interface during early pregnancy and play an important role in vascular remodelling [38]. Impaired maternal spiral artery remodelling is demonstrated in pregnancies complicated by preeclampsia, SGA infants and pre-term birth [3–6].

We found that placental expression of VEGFA, PGF, KDR and FLT1 were significantly reduced in the preeclamptic group compared to the uncomplicated pregnancy group. To our knowledge this is the first study to evaluate placental PGF mRNA expression in pregnancy complications. There is consistent evidence that maternal plasma PGF concentration is decreased as early as the first trimester of pregnancy and that there is a marked reduction prior to the clinical onset of the disease [39]. As the placenta is a major source of PGF during pregnancy, our findings suggest that reduced placental expression and hence production of PGF may contribute to the pathogenic vascular defects seen in preeclamptic pregnancy. A previous study has investigated placental KDR expression in preeclampsia and has demonstrated that expression of both KDR mRNA and protein are decreased in preeclampsia compared to uncomplicated pregnancy [40]. Our finding of reduced KDR mRNA expression in preeclampsia concurs. Consistent with our results, two other groups have shown that placental expression of VEGFA is reduced in preeclamptic placentae compared to normal placentae [19,20] while others have demonstrated an increase [21] and no difference [22,23]. The discrepant results observed among these studies may be due to the differences in the gene expression quantification method, sample size, the many genetic and environmental factors that may alter gene expression and, importantly, phenotypic differences in classification of preeclampsia.

Previously it has been shown that VEGFA immunostaining in the syncytiotrophoblast layer is reduced in growth restricted placentae compared to controls [35]. Consistent with this, we found that the placental expression of VEGFA mRNA was reduced in normotensive SGA pregnancies compared to uncomplicated pregnancy suggesting that the reduced mRNA may correlate with reduced protein expression and contribute to impaired villous vasculogenesis.

We found that VEGFA, PGF, KDR and FLT1 mRNA expression were reduced in placentae from spontaneous pre-term deliveries. At present there is a paucity of data on the role of the VEGF family in pre-term birth, although a failure of physiological transformation of spiral arteries has been reported in this complication [5,6]. A recent study revealed that in the setting of inflammation (elevated high sensitivity C-Reactive Protein), women experiencing pre-term birth had reduced serum PGF levels [41] which is consistent with our findings of reduced placental PGF mRNA expression in the pre-term birth group.

Our finding of reduced expression of VEGFA, PGF and KDR in the gestational hypertension group was surprising considering that placental defects are not established in women with gestational hypertension. However, it is proposed that all these pregnancy complications constitute a continuum of disorders with similar underlying pathogenic abnormalities. The cross talk between fetus, placenta and mother may modulate these effects and the ability of the mother to respond may be a particularly important determinant of outcome.

The VEGF family of angiogenic growth factors gained much interest in the field of preeclampsia research recently due to the consistent and repeated findings of reduced maternal plasma PGF in women destined to develop preeclampsia. There is debate on whether the low level observed is the consequence of low production or the result of inhibition by the endogenous inhibitor sFLT1 which is increased in the maternal circulation in preeclamptic pregnancy. There is debate also on whether the low level of circulating PGF is the cause or the consequence of the disease. VEGFA mRNA expression and protein production are oxygen dependant and are known to be up-regulated by hypoxia [42]. Generally it has been assumed that the placentae in pregnancies complicated by preeclampsia and growth restricted fetuses are hypoxic. If these placentae were hypoxic due to the consequence of the disease, we would expect VEGFA mRNA expression to be up-regulated. We have found that VEGFA mRNA expression is reduced in all these pregnancy complications at term. Potentially their expression was also reduced earlier in gestation and may have contributed to impaired villous angiogenesis. We have recently shown that polymorphisms in the VEGF family genes that are associated with reduced gene expression are also associated with preeclampsia and SGA [43,44]. Inherited susceptibility to reduced placental angiogenic gene expression together with other environmental and life style factors may contribute to the reduced placental expression seen in this study.

The strengths of our study include a well characterized study population, diagnosis of pregnancy complications based on current international guidelines and quantifying gene expression using qRT-PCR. A limitation in our study is that it was not designed to evaluate the expression of VEGF family proteins which would have been beneficial in correlating with the mRNA levels. We also acknowledge that our study groups were relatively small and that the controls were not matched for gestational age. Selection of controls for studies on human placental studies is a vexed issue. Unless placentae are collected from terminations of normal pregnancies in mid to late gestation but prior to 37 weeks, pre-term placentae cannot be considered to be normal. In our hospital we are unable to collect normal placenta earlier than term. However, with the exception of the pre-term group almost all of the
placentae collected for our study were from term (> 37 weeks gestation) pregnancies. Exclusion of the pre-term deliveries from the analyses did not change the results. There was also no relationship between the weight of the placenta and angiogenic gene mRNA expression. Since a fixed amount of RNA was reverse transcribed, this is not surprising. However, lower expression per volume of RNA would translate to lower total mRNA transcription which would be reduced again when the placenta is small. This may contribute to the pathogenesis of pregnancy complications possibly through reduced secretion in to the maternal circulation and maternal maladaptation to pregnancy.

In conclusion, our study demonstrates that placental mRNA expression of VEGF family angiogenic factors is reduced in gestational hypertensive disorders, SGA and in pre-term birth. Identification of this common pathway may be important for future screening and therapeutics.

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Author contributions

PHA contributed to the concept and design of the gene expression study, conducted the laboratory work, conducted the statistical analyses of data and drafted the manuscript. GAD and CTR contributed to the concept and design of the gene expression study and obtained funding; JAL contributed to the laboratory work. All authors critically revised the manuscript for important intellectual content and approved the final version of this manuscript.

Conflicts of interest

None of the authors have any conflicts of interest to declare.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.placenta.2012.02.013

References


