Genetic polymorphisms of vascular endothelial growth factor in severe pre-eclampsia

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Several lines of evidence support the hypothesis that vascular endothelial growth factor (VEGF) plays an important role in the pathogenesis of pre-eclampsia (PE). VEGF is a key component in the regulation of vascular remodelling and the survival of cytotrophoblasts in the placenta. In this case–control study, we aimed to test whether VEGF genetic polymorphisms are associated with the risk of severe PE. We enrolled 84 nulliparous pregnant women with severe PE (PE group). Their VEGF G+405C and VEGF C–2578A genotypes were determined by PCR-restriction fragment length polymorphism (PCR-RFLP) from venous blood samples and were compared with the corresponding VEGF genotypes of 96 nulliparous patients with uncomplicated pregnancies (control group). Carriers of the VEGF+405G allele occurred less frequently in PE than in the control group [P = 0.039; adjusted odds ratio (aOR) = 0.28, range: 0.08–0.93]. Hypertension and proteinuria were diagnosed earlier (by 1.6 weeks and 1.9 weeks, respectively) in PE patients with VEGF–2578A only after adjustment of this association for risk factors of PE. Our results suggest that carriers of VEGF+405G allele have a decreased susceptibility to PE and that the progression of PE may be modified by the presence of VEGF–2578A allele. Nevertheless, the clinical significance of these findings remains to be determined.

Key words: hypertension/polymorphism/proteinuria/severe pre-eclampsia/VEGF

Introduction

Pre-eclampsia (PE) is a multi-organ disorder defined as elevated blood pressure (>140/90 mmHg) and proteinuria that has an onset after the 20th week of gestation. PE affects 5 to 8% of pregnant women depending on the population studied and definitions of PE (ACOG Committee on Practice Bulletins—Obstetrics, 2002; Redman and Sargent, 2005).

Several mechanisms have been suggested to play a role in PE. One of the theories emphasizes the possible role of improper placenta tion. Placenta tion is a complex process that requires the invasion of cytotrophoblasts. As a result, the spiral arteries lose their elasticity, and their diameter increases. Blood flow is increased to satisfy the intensifying demands of the growing fetus (Kaufman et al., 2003). In PE, vascular remodelling is disturbed, probably because of insufficient cytotrophoblast invasion. Therefore, vascular dilatation does not occur in spiral arteries and utero-placental blood flow decreases, leading to local placental hypoxia (Wang and Alexander, 2000).

Vascular endothelial growth factor (VEGF) is a hypoxia-induced growth factor that is produced by cytotrophoblasts. In cultured cytotrophoblasts, increased mRNA and protein levels of VEGF have been observed in response to hypoxia (Li et al., 2005). Zhou et al. found that cytotrophoblast differentiation and invasion during pregnancy is regulated through VEGF receptor-2. They also found that VEGF regulates cytotrophoblast survival and that expression is dysregulated in severe forms of PE (Zhou et al., 2002). VEGF is a component in the regulation of placentation through its effects on vascular remodelling and the survival and invasion of cytotrophoblast.

Several studies have been performed to elucidate its significance in PE. Some authors observed high levels of VEGF in PE that correlate with the severity of the disease (Ong et al., 2000). Several groups have demonstrated that circulating free VEGF concentrations are significantly lower in women with PE (Lyall et al., 1997; Maynard et al., 2003). Levine et al. demonstrated that serum concentration of soluble fms-like tyrosine kinase 1 (sFlt-1), a natural antagonist of VEGF, is increased in PE, and in parallel there is a decrease in the serum level of free VEGF (Levine et al., 2004). These data suggest the potential role of VEGF in the pathogenesis of the disease. In this study, we hypothesized that a maternal genetic component may contribute to altered VEGF production.

The VEGF gene is highly polymorphic. More than 80 single-nucleotide polymorphisms (SNPs) are known in this region of the human genome (NCBI, Gene accession no.: NT 007592). Some of these such as VEGF G+405C and VEGF C–2578A SNPs may have an impact on VEGF production in peripheral blood mononuclear cells (PBMCs) (Watson et al., 2000; Shahbazi et al., 2002) and, presumably, in other tissues. We tested whether these SNPs are associated with the risk and characteristics of severe PE.

Patients and methods

Patients

In this case–control study, we consecutively enrolled 84 nulliparous pregnant women with severe PE at the First Department of Obstetrics and Gynaecology
at Semmelweis University Budapest, Hungary between 1998 and 2004. All participants were of Hungarian ethnicity (Caucasian race). The diagnosis of PE was determined by the American College of Obstetricians and Gynecologists criteria (ACOG Committee on Practice Bulletins—Obstetrics, 2002). Criteria for severe PE were as follows: blood pressure >160/110 mmHg persisting for at least 6 h; and proteinuria ≥5 g/day after the 20th week of gestation. Blood pressure was measured with a mercury sphygmomanometer and the Korotkov sound technique. Diastolic pressure was indicated by the Korotkov sound. All patients had proteinuria ≥3+ or 4+ tested by dipstick in at least two random urine specimens obtained at least 4 h apart or protein excretion rate exceeded 5000 μg/day. Additionally, at least one of the following symptoms was present: headache, visual disturbance, epigastric pain, dyspnea, oliguria (urine ≤40 ml/24 h), thrombocytopenia (≤100 000 μl), elevated concentration of lactate dehydrogenase (LDH) (>600 U/l) and aspartate aminotransferase and alanine aminotransferase activity (AST and ALT ≥70 U/l). There were 12 patients with HELLP syndrome (defined as the common presence of haemolysis, elevated liver enzymes and thrombocytopenia). Patients with chronic hypertension, pre-gestational diabetes and chronic renal disease were excluded. Randomly selected 96 nulliparous women with uncomplicated pregnancies served as controls. Medical history, maternal age, gestational age at delivery and smoking habits were recorded. Pre-pregnancy BMI was calculated as weight in kilograms divided by height in metres squared (for clinical characteristics, see Table I).

The enrolled patients were informed in detail about the study and provided their written consent to collect venous blood samples for diagnostic and scientific purposes including genotyping. The study was approved by the Scientific Ethical Committee of the Semmelweis University (No: 148/1998) in accordance with the declaration of Helsinki.

Genotyping
DNA was extracted with standardized DNA-extracting protocols using Proteinase K enzyme (Roche Diagnostics, Mannheim, Germany). The amount of used blood samples for the DNA extraction was 500 μl. The PCRs were performed in a final volume of 50 μl containing 10% PCR buffer, 2 mM MgCl₂, 0.2 mM dNTPs, 1.5 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and sense and antisense primers, 0.5 μM of each. We used 100 ng DNA per PCR reaction. The investigated DNA sequences were amplified by the following primers: VEGF C–2578A: forward: 5’-GGGCGTTAGGACACGATC-3’ and reverse: 5’-TGCCCCAGGGAACAAAGT-3’; VEGF G+405C SNP: forward: 5’-CGGCGGTCACCCCCAAAAG-3’ and reverse: 5’-CGCCGGTGACCCCTGGCAGATG-3 reverse and reverse: 5’-CGCCGGTGACCCCTGGCAGATG-3 reverse.

The condition of PCRs was as follows: 20 s at 94°C (denaturing), 20 s at 65°C (annealing), 30 s at 72°C (extension) for 40 cycles. For the VEGF C–2578A, the resultant 267bp length product was subsequently digested by Bgl II restriction endonuclease (Fermentase Inc., Hanover, MD, USA) at 37°C overnight. This resulted in two fragments 208 and 60 bp in the presence of VEGF +405G allele. The PCR products and digested PCR products were then run on 3% agarose gel followed by ethidium bromide staining. Determination of VEGF G+405C genotype was not successful in three cases.

Data analysis
Hardy–Weinberg equilibrium of the tested SNPs were calculated using the Arlequin software (http://embnet.unige.ch/arlequin). Logistic regression analysis was used for the analysis of the association between VEGF genotype and risk of severe PE. Multiple linear regression analysis was used to test the association between carrier state of VEGF genotypes and haplotypes and diagnosis date of hypertension and proteinuria. The associations were adjusted for maternal age, BMI before pregnancy and smoking during pregnancy (Lain et al., 1999; Duckitt and Harrington, 2005). All calculations were performed with the statistical software package SPSS 10.0.

Results
The genotype distribution of the investigated VEGF SNPs fulfilled Hardy–Weinberg criteria in the PE and control groups. Table I summarizes the genotype distribution of VEGF G+405C and VEGF C–2578A polymorphisms.

The results of logistic regression analysis indicated that heterozygous and homozygous carrier states of VEGF+405G alleles presented an independent protective factor in severe PE ($P = 0.039$, OR: 0.28 (0.08–0.93)]. This association was adjusted for the risk factors of PE (maternal age, BMI before pregnancy and smoking during pregnancy) (Lain et al., 1999; Duckitt and Harrington, 2005) (for details, see Table II).

Multiple linear regression analysis revealed that in carriers of VEGF C–2578A allele, hypertension ($P = 0.014, B = -1.64, \beta = -0.27$) and proteinuria ($P = 0.03, B = -1.89, \beta = -0.34$) were diagnosed earlier (by 1.6 and 1.9 weeks, respectively) (Table III). Hypertension and proteinuria were diagnosed later in carriers of VEGF+405G/C–2578A CC haplotype by 2.8 ($P = 0.001, B = 2.77, \beta = 0.37$) and 1.8 weeks ($P = 0.027, B = 1.78, \beta = 0.25$), respectively (data not shown). The associations were adjusted for risk factors enlisted above. (‘B’ refers to the raw, whereas ‘β’ refers to the standardized regression coefficient.)

Discussion
Our results suggest that the presence of the VEGF+405G allele in nulliparous pregnant women is associated with a decreased risk of severe PE. The association is independent of examined risk factors of PE.

| Table I. Allele frequencies of VEGF G+405C and VEGF C–2578A polymorphisms of pre-eclamptic women and control group |
|----------------|----------------|----------------|----------------|
| Allele frequencies of VEGF G+405C polymorphisms | PE (n = 84) | Controls (n = 96) | Adjusted OR (95% CI) |
| VEGF+405G allele | 97 (58) | 122 (64) | 0.28 (0.08–0.93)* |
| VEGF+405C allele | 71 (42) | 70 (36) | 1.31 (0.6–2.82) |
| Total allele | 168 | 192 | – |
| Allele frequencies of VEGF C–2578A polymorphisms | PE (n = 84) | Controls (n = 96) | Adjusted OR (95% CI) |
| VEGF–2578C allele | 108 (62) | 108 (56) | 1.02 (0.4–2.62) |
| VEGF–2578A allele | 66 (38) | 86 (44) | 0.55 (0.25–1.21) |
| Total allele | 174 | 192 | – |

OR, odds ratio; PE, pre-eclampsia; VEGF, vascular endothelial growth factor. *Adjusted for maternal age, mother’s BMI before pregnancy and smoking during pregnancy.
Clinical characteristics and vascular endothelial growth factor (VEGF) genotypes in women with pre-eclampsia

(maternal age, BMI before pregnancy and smoking during pregnancy). Furthermore, among nulliparous pregnant women with severe PE, carrier state of the VEGF–2578 A allele may be associated with the accelerated development of disease.

Until now, only one study has dealt with the association of VEGF SNPs and the risk of PE. Papazoglou et al. demonstrate an association between the most severe form of PE and the VEGF C<sup>936T</sup> SNP. They also investigated VEGF G<sup>405C</sup> and VEGF C<sup>–2578A</sup> polymorphisms; however, there was no significant association between these SNPs and PE (Papazoglou et al., 2004). Probably, the explanation for the different results is the different study design. Papazoglou et al. enrolled post-menopausal women with uncomplicated pregnancies in their medical history, whereas we used nulliparous women with healthy pregnancy as controls. As age may be a confounding factor in genetic epidemiological studies (Aramon et al., 2003), to avoid this bias, we selected an age-matched control group.

Recently, it has been revealed that the VEGF G<sup>405C</sup> and VEGF C<sup>–2578A</sup> polymorphisms determine the production of VEGF. Significant correlation was observed between the VEGF G<sup>405C</sup> and VEGF C<sup>–2578A</sup> SNPs and the VEGF production of lipopolysaccharide-induced PBMCs. Highest VEGF production was associated with the VEGF<sup>G405C</sup>/GG genotype; intermediate production was associated with the GC genotype and lowest production with the CC genotype (Watson et al., 2000). In the case of the VEGF C<sup>–2578A</sup> SNP, the CC homozygous PMBCs produced significantly more VEGF than AA homozygous cells (Shahbazi et al., 2002).

Our results suggest that genetic polymorphisms of VEGF gene, linked to an inherited alteration of VEGF production, may contribute to the pathogenesis of PE. We found that carrier state of the VEGF<sup>G405C</sup> allele, which is accompanied by high VEGF-producing capability, decreases the risk of severe PE. VEGF has a central role in many processes that are involved in the development and progression of PE. VEGF is known to play a role in the regulation of cytotrophoblast invasion and placentation (Zhou et al., 2002). It could be hypothesized that our observation that an inherited increase of VEGF-producing ability could be protective against PE is in relation to the earlier experience that VEGF has a potential effect on placentation.

As hypertension and proteinuria were diagnosed earlier in the presence of the VEGF<sup>–2578A</sup> allele, we also speculate that the progression of PE may be accelerated in the presence of polymorphisms which predispose to low VEGF-producing capacity.

Several lines of evidence support that VEGF has an impact on the regulation of systemic blood pressure. VEGF is known to induce endothelium-dependent vasodilatation in vivo (Horowitz et al., 1997). Systemic administration of VEGF causes hypertension in a dose-dependent manner (Yang et al., 1996). The VEGF-induced hypertension is probably mediated by nitric oxide (Lopez et al., 1997) and prostacyclin (He et al., 1999). In line with these data, we found association between the earlier onset of hypertension and the carrier state of the VEGF<sup>–2578A</sup> allele, which decreases the VEGF-producing capability (Shahbazi et al., 2002).

VEGF also has an important role in maintaining the integrity of the glomerular filtration barrier, as inhibition of VEGF activity may lead to proteinuria (Eremina et al., 2003; Bdlolah et al., 2004). This effect of VEGF in the regulation of glomerular filtration may account for the association between the earlier onset of proteinuria and the carrier state of the VEGF<sup>–2578A</sup> allele, which predisposes to low production of VEGF (Shahbazi et al., 2002).

Even though the hypertension and proteinuria were diagnosed earlier in the presence of the VEGF<sup>–2578A</sup> allele after adjustment of risk factors, the clinical significance of this result could be queried, and further investigation is needed to establish it.

It is tempting to speculate that differing VEGF production is the reason for these associations. Our case–control analysis is not suitable for testing this theory. The causative role of these genetic variants in severe PE should be tested by determining VEGF levels in different genotypes of VEGF G<sup>405C</sup> and VEGF C<sup>–2578A</sup> both in the healthy reference population and in women with severe PE.

It is also conceivable that the observed association is not directly related to VEGF production capacity. The VEGF gene is located near to the major histocompatibility complex (MHC) region on chromosome 6. The genes of several other proteins that are possibly implicated in PE [e.g. heat shock protein 70 (Jirecek et al., 2002), tumour necrosis factor (TNF)-α (Conrad and Benyo, 1997)] are also located here. Therefore, it is possible that the observed association between severe PE and VEGF genotypes is the result of linkage disequilibrium with other neighbouring gene polymorphisms.

In conclusion, we found that the presence of the VEGF<sup>G405C</sup> allele in nulliparous pregnant women is associated with a decreased risk of severe PE. Furthermore, our results suggest that in addition to the risk, the progression of severe PE may also be modified by VEGF polymorphisms; however, to confirm this hypothesis, further prospective studies are needed.

**Table III. Clinical characteristics and vascular endothelial growth factor (VEGF) genotypes in women with pre-eclampsia**

<table>
<thead>
<tr>
<th>VEGF G&lt;sup&gt;405C&lt;/sup&gt; (n = 84)</th>
<th>VEGF C&lt;sup&gt;–2578A&lt;/sup&gt; (n = 87)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diagnosis of hypertension</strong></td>
<td><strong>Diagnosis of proteinuria</strong></td>
</tr>
<tr>
<td>(gestational weeks)</td>
<td>(gestational weeks)</td>
</tr>
<tr>
<td>GG (n = 31)</td>
<td>CC (n = 36)</td>
</tr>
<tr>
<td>31.6 ± 3</td>
<td>31.9 ± 3.1</td>
</tr>
<tr>
<td>GC (n = 35)</td>
<td>CA (n = 36)</td>
</tr>
<tr>
<td>29.5 ± 3.3</td>
<td>29.6 ± 3.2</td>
</tr>
<tr>
<td>CC (n = 18)</td>
<td>AA (n = 15)</td>
</tr>
<tr>
<td>29.9 ± 2.2</td>
<td>29.4 ± 1.4</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD; Boldface characters signify multiple linear regression analysis. P < 0.05. Results are adjusted for risk factors of pre-eclampsia.

References


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