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Vascular endothelial growth factor gene polymorphisms and pre-eclampsia

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Vascular endothelial growth factor (VEGF) plays a crucial role in physiological vasculogenesis and vascular permeability and has been implicated in the pathogenesis of pre-eclampsia. Our present study was undertaken to identify associations between three functional VEGF gene polymorphisms, linked with altered VEGF gene responsiveness, and pre-eclampsia. The study involved 42 pre-eclamptic and 73 healthy control women who were genotyped for the –2578C/A, –634G/C and 936C/T polymorphisms of the VEGF gene. No significant association between genotypic or allelic frequencies in women with pre-eclampsia relative to controls was found. A statistically significant difference was found for allelic frequencies of the 936C/T polymorphism between women with severe pre-eclampsia and controls (odds ratio: 2.70; 95% confidence interval: 1.09–6.63; *P* = 0.019). VEGF gene polymorphisms studied are unlikely to be major predisposing factors for pre-eclampsia. The presence of the 936T allele probably has a considerable effect on disease modification.

Key words: pre-eclampsia/polymorphisms/vascular endothelial growth factor

Introduction

Pre-eclampsia (PE) is a multisystem disorder that is unique to human pregnancy and remains a major cause of maternal and fetal morbidity and death (Murphy and Stirrat, 2000). Susceptibility gene studies of PE so far have been based upon the currently most widely accepted hypotheses on its aetiology and pathogenesis. These hypotheses comprise placental ischaemia, oxidative stress and maternal–fetal immune maladaptation (Dekker and Sibai, 1998). These are all thought to be associated with the two key features of the clinical syndrome of PE; shallow endovascular trophoblast invasion in the spiral arteries in early pregnancy and generalized endothelial cell dysfunction (Dekker and Sibai, 1998).

Vascular endothelial growth factor (VEGF) is a major angiogenic factor and is a prime regulator of endothelial cell proliferation. It plays a crucial role in physiological vasculogenesis and vascular permeability (Ferrara *et al*., 1992). The gene encoding VEGF is located on chromosome 6 band p21 and comprises a 14 kb coding region with 8 exons and 7 introns (Vincenti *et al*., 1996). It binds to Flt-1 (also named VEGFR1) and to flk1 (also named KDR VEGFR2), both tyrosine kinase receptors present on endothelial cell membranes (de Vries *et al*., 1992; Terman *et al*., 1992). VEGF (or VEGF-A) belongs to a gene family that includes placental growth factor (PLGF), VEGF-B, VEGF-C and VEGF-D. They share structural features typical of the VEGF family, but display different biological activities, mainly owing to their different specificities for the known VEGF receptors. Homologues of VEGF have also been identified in the genome of the parapoxvirus Orf virus and shown to have VEGF-like activities (Ferrara *et al*., 2003).

Expression of VEGF mRNA is rapidly and reversibly induced by hypoxia both *in vitro* and *in vivo* (Minchenko *et al*., 1994; Gargett *et al*., 2001). Its expression is also up-regulated in both physiological and pathological states where increased angiogenesis occurs (Gargett *et al*., 2001). VEGF and its receptors may play a pivotal role in the altered function of PE (Brockelsby *et al*., 1999). Several groups have demonstrated that circulating total VEGF concentrations are significantly elevated in women with PE (Baker *et al*., 1995; Kupferminc *et al*., 1997; Hunter *et al*., 2000). Free (unbound) VEGF concentrations have been reported to be significantly lower (Lyall *et al*., 1997; Maynard *et al*., 2003). This inconsistency is probably due to highly elevated levels of the soluble fms-like tyrosine kinase 1 (sflk1) receptor which captures free VEGF (Maynard *et al*., 2003). A positive correlation between VEGF concentrations and systemic or uterine vascular resistance has been described (Simmons *et al*., 2000) and a negative correlation between VEGF and the stable nitric oxide metabolite nitrate (El-Salahy *et al*., 2001) has been demonstrated in women with PE. Because patients who are predestined to have PE demonstrate increased concentrations of VEGF before the clinical onset, VEGF has been suggested to be involved in the pathogenesis of PE rather than being an effect of the disease (Hunter *et al*., 2000; Simmons *et al*., 2000; El-Salahy *et al*., 2001). The VEGF system is up-regulated, both at the transcriptional and translational level, in response to chronic placental hypoxia (PE) (Trollmann *et al*., 2003).

Moreover, it has been demonstrated that mice lacking one VEGF allele in renal podocytes develop the typical renal pathology found in pregnant women with PE, which comprises additional evidence for a critical role of VEGF in renal disease during PE (Eremina *et al*., 2003).

Many polymorphisms of the VEGF gene have been identified so far. A few of them have been correlated with variation in VEGF protein production (Brogan *et al*., 1999; Renner *et al*., 2000; Watson *et al*., 2000; Awata *et al*., 2002). In the present study we investigated whether there is any association between PE and three common functional VEGF single nucleotide polymorphisms, –2578C/A in the promoter region (Brogan *et al*., 1999), –634G/C in the 5′- untranslated region (Awata *et al*., 2002) and 936C/T in the 3′-untranslated region (Renner *et al*., 2000).

Materials and methods

The study included a total number of 115 women (42 with PE and 73 controls). Patients with PE were recruited at the Department of Obstetrics and Gynecology, Democritus University of Thrace, General Hospital of Alexandroupolis. PE was defined as systolic blood pressure of ⩾140 mmHg and/or diastolic blood pressure of ⩾90 mmHg on two occasions ⩾6 h apart after 20 weeks of gestation, but before the onset of labour, plus proteinuria of ⩾2+ (dipstick method) or ⩾0.3 g/24 h (American College of Obstestricians and Gynecologists, 1996). Severe pre-eclampsia was defined as a higher blood pressure ⩾160 mmHg systolic or ⩾110 mmHg diastolic on two occasions ⩾6 h apart, and a proteinuria level ⩾5 g/24 h or ⩾3+ by dipstick testing on at least two separate occasions (American College of Obstestricians and Gynecologists, 1996). According to the above criteria, 22 patients had mild, while 20 patients had severe, pre-eclampsia. The control group consisted of 73 post-menopausal women with a history of at least two uncomplicated pregnancies. Demographic data of participants and clinical characteristics of patients are shown in Table I. All participants were of Greek ethnicity. Women with chronic hypertension were excluded from the study. Written informed consent was obtained from all participating women.

DNA was isolated from 200 µl of anticoagulated peripheral blood using a commercially available kit according to the manufacturer's instructions (QIAamp DNA Blood Mini Kit; Qiagen Inc., USA). Amplification of the three regions of the VEGF gene, containing the polymorphisms –2578C/A, –634G/C and 936C/T, were carried out in a Mastercycler gradient (Eppendorf–Netheler–Hinz GmbH, Germany) thermal cycler in 20 µl reaction volumes containing 20 mmol/l Tris–HCl (pH 8.4), $MgCl_2$, 50 mmol/l KCl, 0.2 mmol/l of each nucleotide, 20 pmol of each of the forward and reverse primers, 1 U Platinum *Taq* polymerase (Gibco–BRL, USA) and 500 ng of DNA. $MgCl_2$ concentrations were optimized for each amplification (2.75 mmol/l for –2578C/A, 4 mmol/l for –634G/C and 4.5 mmol/l for 936C/T). Following an initial denaturation step (5 min at 94°C), samples were subjected to 35 rounds of PCR consisting of 94°C for 40 s, 62°C (–2578C/A), 58°C (–634C/G) or 64°C (936C/T) for 1 min; and 72°C for 40 s with a final extension time of 5 min at 72°C. For the –2578C/A polymorphism the following specific/common primers were used, generating a PCR product of 77 bp: 5′-TAGGCCAGACCCTGGCAC-3′ (C polymorphism) or 5′-TAGGCCAGACCCTGGCAA-3′ (A polymorphism) with 5′-TGCCCCAGGGAACAAAGT-3′. For the –634G/C the following primers amplified a fragment of 304 bp: forward 5′-ATTTATTTTTGTCTGTCTGTCTGTCCGTCA-3′ and for the 936C/T the following primers amplified a fragment of 208 bp: forward 5′-AAGGAAGAGGAGACTCTGCGCAGAGC-3′, reverse 5′-TAAATGTATGTATGTGGGTGGGTGTGTCTACAGG-3′. The VEGF –634G/C polymorphism was analysed by digestion of the PCR product with restriction endonuclease *BsmF*I (New England Biolabs, USA). The –634G allele was cut into two fragments of 193 and 111 bp while the –634C allele remained uncut (304 bp). The VEGF 936C/T polymorphism was analysed by digestion of the PCR product with restriction endonuclease *Nla*III (New England Biolabs). The 936C allele remained uncut (208 bp), while the 936T was cut into two fragments of 122 and 86 bp. PCR products and restriction fragments were analysed by electrophoresis through 2.0% agarose gels, stained with ethidium bromide, and photographed.

Table I. Clinical and demographic characteristics of pre-eclamptic women and controls

	Controls (n = 73)	Pre-eclamptic women		
		Total (n = 42)	Mild (n = 22)	Severe (n = 20)
Age (years, mean ± SD)	53.4 ± 8.2	25.2 ± 5.3	24.5 ± 4.3	25.2 ± 5.3
Gravidity (median, range)	3 (2–5)	2 (1–3)	2 (1–3)	2 (1–2)
Parity (median, range)	2 (2–5)	1 (0–2)	1 (0–2)	1 (0–2)
Gestational age[a] (weeks)		36.4 (34.9–38.3)	37.2 (35.3–38.3)	35.6 (32.9–38.1)
Diastolic blood pressure[a] (mmHg)		149 (145–165)	145 (142–162)	162 (161–168)
Systolic blood pressure[a] (mmHg)		102 (96–106)	100 (97–102)	103 (101–107)

[a]Values are expressed as median and interquartile range (25th–75th percentiles).

Table II. Genotype frequencies of –2578A/C, –634G/C and 936C/T polymorphisms among women with pre-eclampsia (n = 42) and controls (n = 73)

Genotypes	Pre-eclampsia n (%)	Controls n (%)	P
–2578A/C			A versus B: 0.42; A versus C: 0.79; B versus C: 0.26; A, B versus C: 0.37
(A) *AA*	10 (23.8)	20 (27.4)	
(B) *AC*	22 (52.4)	30 (41.1)	
(C) *CC*	10 (23.8)	23 (31.5)	
–634G/C			A versus B: 0.79; A versus C: 0.87; B versus C: 0.90; A, B versus C: 0.99
(A) *CC*	10 (23.8)	16 (21.9)	
(B) *GC*	17 (40.5)	31 (42.5)	
(C) *GG*	15 (35.7)	26 (35.6)	
936C/T			A versus B: –; A versus C: –; B versus C: 0.09; A, B versus C: 0.09
(A) *TT*	1 (2.4)	1 (1.3)	
(B) *CT*	15 (35.7)	16 (22)	
(C) *CC*	26 (61.9)	56 (76.7)	

Differences in the VEGF genotype and allele frequencies between the study and control groups were analysed by χ^2-test or Fisher's exact test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed by using standard software (V10, SPSS for MS Windows).

Results

We examined genotype and allele frequencies of three common single nucleotide polymorphisms of the vascular endothelial growth factor gene in 42 women with PE and 73 controls. Genotypes were found to be in Hardy–Weinberg equilibrium in both the study and control groups. Genotypic and allelic frequencies of cases and controls are shown in Tables II and III respectively. There was no significant association between genotypic or allelic frequencies in women with pre-eclampsia relative to controls. Additional subgroup analyses (mild and severe pre-eclampsia) revealed a statistically significant difference ($P = 0.019$) for allelic frequencies of the 936C/T polymorphism between women with severe pre-eclampsia and controls (Table IV) but not for the other polymorphisms studied (data not shown).

Discussion

Family studies have shown that genetic factors play a role in pre-eclampsia but the exact inheritance pattern is still unknown (Chesley *et al*., 1986; Arngrimsson *et al*., 1995). Studies investigating the effects of plasma/serum from women with pre-eclampsia suggest that there are several characteristics that any candidate factor(s) must possess. These include the ability to pass freely into the maternal circulation, increase cellular permeability and cell turnover and alter prostacyclin and NO production (Hayman *et al*., 1999). VEGF is one such candidate as it possesses the characteristics of all of the above.

In this study we attempted to establish an association between three common functional polymorphisms of the VEGF gene and PE. We did not find any significant association between these polymorphisms and the occurrence of PE, but a limitation of our study is in its relatively small sample size which theoretically increases the likelihood of a type II error. Another concern relates to the selection of a proper control group. Studies investigating women with pre-eclampsia usually use age-matched controls to compare genotype frequencies. This strategy does not rule out a possible future pre-eclamptic pregnancy. To avoid this possible bias, all control women were post-menopausal at the time of blood sampling. A statistically significant correlation was found between the 936C/T polymorphism and the severity of PE.

The role of VEGF in pre-eclampsia has received substantial attention. Several authors have reported increased systemic VEGF levels in women with PE (Baker *et al*., 1995; Kupferminc *et al*., 1997; Hunter *et al*., 2000) whereas other authors have reported decreased levels (Lyall *et al*., 1997; Maynard *et al*., 2003), a discrepancy which probably has to do with the methodology (Maynard *et al*., 2003). In pregnancy, as compared to the non-pregnant state, most VEGF is bound to circulating sFlt1 due to very high levels of the latter. There is circumstantial evidence that antagonism of VEGF may have a role in hypertension and proteinuria (Koga *et al*., 2003; Maynard *et al*., 2003). Free VEGF levels, which more accurately reflect effective circulating VEGF, are substantially lower than total VEGF levels. It seems therefore that PE is characterized by normal to high total VEGF levels (perhaps induced by placental hypoxia) but low free VEGF levels, owing to a vast excess of sFlt1 which antagonizes the VEGF effects on the formation of placental vasculature and maternal endothelial cell function (Maynard *et al*., 2003). It should also be mentioned that sFlt1 acts through its antagonism of both VEGF and placental growth factor (PlGF), since the VEGF antagonist sFlk1 does not produce the pre-eclampsia phenotype in pregnant rats (Maynard *et al*., 2003). The origin of the elevated VEGF levels has not been confirmed conclusively. Conflicting results have been reported regarding the regulation of the VEGF system in PE at the transcriptional and translational level in the placenta (Cooper *et al*., 1995; Ranheim *et al*., 2001; Geva *et al*., 2002; Trollmann *et al*., 2003). Most candidate genes and all genome-wide scans so far have focused mainly on maternal genetic factors, but evidence is growing that the fetal gene load influences a mother's susceptibility to pre-eclampsia (Lachmeijer *et al*., 2002). Seen in this light, measuring VEGF levels in the umbilical vein and artery and investigating maternal and fetal VEGF polymorphisms would be more informative about possible associations between VEGF and PE.

We found a statistically significant correlation between the 936C/T polymorphism and the severity of PE. Subjects carrying the T allele have significantly lower VEGF plasma levels than subjects carrying the VEGF 936*CC* genotype (Renner *et al*., 2000). Interestingly, there is an independent association between plasma VEGF concentration and plasma PlGF concentration and pre-eclampsia (Livingston *et al*., 2000) and the clinical severity of pre-eclampsia seems to correlate with the magnitude of various cytokine abnormalities including VEGF (Madazli *et al*., 2003). It has also been demonstrated that second-trimester analysis of circulating VEGF appears to be a useful tool for the early identification of pregnant women at increased risk for developing severe, early-onset PE (Polliotti *et al*., 2003).

Table III. Allelic frequencies of –2578A/C, –634G/C and 936C/T polymorphisms among women with pre-eclampsia (PE) and controls

Alleles	PE *n* (%)	Controls *n* (%)	*P*
–2578A/C			0.76
A	42 (50)	70 (47.9)	
C	42 (50)	76 (52)	
A/C ratio	1.0	0.92	
–634G/C			0.69
C	34 (40.7)	63 (43.1)	
G	50 (59.5)	83 (56.8)	
C/G ratio	0.68	0.73	
936C/T			0.1
T	17 (20.2)	18 (12.3)	
C	67 (79.7)	128 (87.6)	
T/C ratio	0.25	0.15	

Table IV. Allelic frequencies of the 936C/T polymorphism among controls and women with severe or mild pre-eclampsia (PE)

Alleles	Mild PE (44 alleles)	OR (95% CI)	Severe PE (40 alleles)	OR (95% CI)	Controls (146 alleles)
T	5	0.91 (0.31–2.61)	11	2.70 (1.09–6.63)	18
C	39	$P = 0.86$	29	$P = 0.019$	128

OR = odds ratio; CI = confidence interval.

In summary, it seems plausible that angiogenic molecules such as VEGF, PlGF and Flt1 may be important regulators of early placental development and pseudovasculogenesis. Although the power of this study was limited, we conclude that the VEGF gene polymorphisms studied are unlikely to be major predisposing factors for pre-eclampsia. Nevertheless, taking into account that VEGF has an important role during pregnancy, the present data strongly suggest that a reduced ability of mother's tissue to up-regulate VEGF (as predicted by the 936C/T VEGF polymorphism) has a considerable effect on disease modification. Further studies should investigate whether other genetic alterations could predict this catastrophic disease early in pregnancy or even before conception.

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