Genetic polymorphisms for vascular endothelial growth factor in perinatal complications

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ABSTRACT. Low birth weight (LBW) infants have increased susceptibility to perinatal complications. An immature and impaired vascular system may possibly participate in these complications. There is evidence that supports the notion that vascular endothelial growth factor (VEGF), which is an essential regulator of embryonic angiogenesis, plays a central role in the pathogenesis of perinatal complications. We aimed to test whether functional genetic polymorphisms of VEGF are associated with the risk of preterm birth or perinatal morbidity. We enrolled 128 LBW infants ($\leq$ 1500 grams). VEGF T-460C, VEGF C-2578A and VEGF G+405C polymorphisms were determined by real-time PCR or PCR-RFLP, respectively. Their genotypes were compared with VEGF genotypes of 200 healthy, term neonates. The prevalence of the VEGF+405 C allele was higher in LBW infants than in healthy, term neonates (OR [95% CI]: 1.29 [1.01-1.65]). Carrier state for the VEGF -2578A allele was an independent risk factor for enterocolitis necrotisans (NEC) (adjusted OR [95% CI]: 2.77 [1.00-7.65]). The carrier state for the VEGF -2578AA genotype was associated with a decreased risk of acute renal failure (ARF) (adjusted OR [95% CI]: 0.2 [0.05-0.78]). These results suggest that VEGF G+405C polymorphism might be associated with a higher risk of preterm birth and that VEGF C-2578A polymorphism may participate in the development of perinatal complications such as NEC and ARF.

Keywords: genetic polymorphism, low birth weight infant, perinatal morbidity, vascular endothelial growth factor

In industrialized countries, 5-11% of infants are born pre-term (< 37 weeks' gestation), and because of perinatal complications, preterm births account for up to 75% of neonatal morbidity [1].

In LBW infants, the immature and impaired vascular system plays a central role in the pathogenesis of the most common perinatal complications such as intraventricular hemorrhage (IVH), retinopathy of prematurity (ROP), congenital heart disease, bronchopulmonary dysplasia (BPD) [2], acute renal failure (ARF) [3] and enterocolitis necrotisans (NEC) [4].

The significance of vascular endothelial growth factor (VEGF) has been recognized in the pathogenesis of these perinatal complications. VEGF is a potent vascular perme-ability and angiogenic factor which regulates multiple endothelial cell functions, such as apoptosis and synthesis of nitric oxide and prostacyclin [5].

A significant association was demonstrated between con-centrations of VEGF in cerebrospinal fluid and the devel-opment of post-hemorrhagic hydrocephalus after severe IVH [6]. It was observed that during the first phase of ROP, the production of VEGF was decreased [7], while it was increased during the second, proliferative, phase of the disease [8]. Decreased VEGF mRNA expression was found in the lung of human, premature newborns dying of BPD [9]. It is also known that VEGF plays a role in heart morphogenesis including heart septation [10,] and it is important in the vascular remodelling occurring during ductus arteriosus closure [11]. Although there are no hu-man data about the role of VEGF in acute renal failure in premature infants, it was revealed in an animal experiment, that anti-VEGF therapy inhibits glomerular development in newborn mice [12]. These data suggest a potential role for VEGF in the pathogenesis of perinatal complications.

The VEGF gene is highly polymorphic. Some of these polymorphic loci have been identified as determinants of VEGF production. A significant correlation was observed between genotypes for VEGF G+405C, VEGF C-2578A SNPs and the production of VEGF in peripheral blood mononuclear cells [13, 14]. It was also revealed that the common carrier state of VEGF- 460C and VEGF+405G alleles in the promoter have a 71% higher basal promoter activity when compared with the wild-type sequence [15]. In previous studies, we found correlations between VEGF G+405C polymorphism and the risk of ROP [16], in addi-tion to the risk of congenital heart disease [17].

Assuming that these polymorphisms could alter the VEGF producing ability, in the present study we tested whether the carrier states for VEGF C-2578A, VEGF T-460C and VEGF G+405C are associated with the risk of preterm birth and perinatal complications.

PATIENTS AND METHODS

Patients

We enrolled 128 LBW (≤ 1500 grams) infants and 200 healthy, term infants in our retrospective study. All of the infants were delivered, and LBW infants were treated, at the Second Department of Gynaecology and Obstetrics, Semmelweis University, Budapest.

The mean gestational age of the LBW infants was 30 ± 3 weeks and the mean birth weight was 1170 ± 280 grams. One hundred and eleven infants were single births and 17 were one sibling of 17 twins enrolled. We made subgroups according to the presence of perinatal complications such as respiratory distress syndrome (RDS) (n = 60) NEC (n = 49), IVH (n = 42), ARF (n = 41), BPD (n = 23), persistent ductus arteriosus (PDA) (n = 47) and atrial septal defect (ASD) (n = 13). Perinatal complications were defined according to internationally accepted criteria [18-22].

We compared the genotype distributions of LBW infants with the healthy, term neonates. The mean of gestational age of the healthy, term neonates was 39±1.1 weeks and the mean birth weight was 3400±390 grams. We also compared the genotype distributions of each perinatal complication subgroup with those LBW infants who did not suffer any diseases (control groups). The study was approved by an Institutional Ethical Committee (TUKEB 14/2003).

Genotyping

Dried blood samples taken for metabolic screening after the 5th postnatal day or at the start of oral feeding were used for genotyping. DNA was extracted using an agent (Chelex®, BioRad, Germany) according to the manufacturer's instructions.

VEGF C-2578A and VEGF G+405C SNPs were detected using the PCR-RFLP method. The PCRs were performed in a final volume of 50 μl containing 10% 10x PCR buffer, 2mM $MgCl_2$, 0.2 mM dNTPs, 1.5 U Taq DNA polymerase (Invitrogen, California, USA) and sense and antisense primers, 0.5 μM of each. The specific primer pairs for VEGF C-2578A and G+405C were as follows: forward: 5'-GGGCCTTAGGACACCATACC-3' and reverse: 5'-TGCCCCAGGGAACAAAGT-3' and forward: 5'-CCGACGGCTTGGGGAGATTG-3 and reverse: 5'-CGGCGGTCACCCCCAAAAG-3', respectively. The amplification profile consisted of denaturation at 94 °C for 20s, annealing 57 °C (VEGF C-2578A) or 60 °C (VEGF G+405C) for 30 s and extension at 72 °C for 30s for 40 amplification cycles. The resultant 267 bp (VEGF C-2578A) and 197 bp (VEGF G+405C) length products were digested by Bgl II (VEGF C-2578A) and BsmF I (VEGF G+405C) at 37 °C or at 65 °C overnight, respectively.

VEGF T-460C SNP was determined by real-time PCR quantification using fluorescence resonance energy transfer (FRET) hybridisation probes on a Light Cycler system (Roche Diagnostics, Mannheim, Germany). Primers and probes were as follows: forward primer: 5'-AGACGGCAGTCACTAG-3'; reverse primer: 5'-AATATTGAAGGGGGCAG-3'; one probe (5'-AGCGGGGAGAAGGCCAGGG-3') has been labelled at the 5' end with the Light Cycler Red 640 fluorophore; the other one (5'-TGTGGGGTTGAGGGCGTT-3') has been labelled at the 3' end with fluorescein (Tibmolbiol, Berlin, Germany).

Data analysis

Hardy-Weinberg equilibria of the VEGF C-2578A, VEGF T-460C and VEGF G+405C SNPs were calculated using the Arlequin software (http://lgb.unige.ch/arlequin/).

For statistical analysis, the chi-square test was used to compare allelic and genotype frequencies in LBW infants and healthy, term neonates, in LBW infants with each perinatal complication and in corresponding control groups. For the adjustment for known risk factors of each perinatal complication, logistic regression analysis was used to compare allelic and genotype frequencies between LBW infants with each perinatal complication and corresponding control groups. The level of significance was set at p < 0.05. All calculations were performed with the statistical software package SPSS 10.0.

RESULTS

The genotype distribution of the VEGF C-2578A, VEGF T-460C and VEGF G+405C SNPs fulfilled the Hardy-Weinberg criteria in all groups (in LBW infants and healthy, term neonates and in LBW infants with each perinatal complication, and control groups).

table 1 summarizes the allele frequency and genotype distribution of VEGF C-2578A, VEGF T-460C and VEGF G+405C polymorphisms in LBW infants and healthy, term neonates. The prevalence of the VEGF +405 C allele was higher in LBW infants than in healthy, term neonates (p = 0.046, odds ratio [95% CI]: 1.29 [1.01-1.65]).

The prevalence of the VEGF -2578A alleles was higher in LBW infants with NEC compared with the corresponding control group (p = 0.049, adjusted odds ratio [95% CI]: 2.77 [1.00-7.65]). This result was adjusted for gestational age, sepsis, PDA and cardiac failure (CF) (table 2).

We also found that carriers of the VEGF -2578AA genotype were significantly fewer among the LBW infants with ARF than in the corresponding control group (p = 0.021, adjusted odds ratio [95% CI]: 0.2 [0.05-0.78]); adjusted for gestational age, sepsis, NEC, RDS, PDA, CF (table 2).

DISCUSSION

VEGF is a key regulator of vascular permeability and angiogenesis, and regulates multiple endothelial cell functions. VEGF induces endothelial cell proliferation [23], migration [24] and differentiation [25], and stimulates endothelial cell survival [26]. Owing to its angiogenic and mitogenic properties, VEGF is essential in embryonic de-

Table 1

Vascular endothelial growth factor (VEGF) genetic polymorphisms in LBW infants and in healthy, term neonates. Birth weight and gestational age are presented as mean±SD

	Low birth weight infants	Healthy term neonates
N	128	200
Gender (male/female)	6/62	99/101
Birth weigth mean (SD) grams	1170 ± 280	3400 ± 390
Gestional age mean (SD) weeks	30 ± 3	39 ± 1.1
VEGF C^{2578} A genotypes		
CC/CA/AA	28/60/26	39/91/43
prevalence of A allele	49	51
VEGF T^{460} C genotypes		
TT/TC/CC	31/68/29	19/64/30
prevalence of C allele	49	55
VEGF G^{+405} C genotypes		
GG/GC/CC	58/56/7	75/38/8
prevalence of C allele	29*	22

* Chi-square test: p=0.046, odds ratio [95%CI]: 1.29[1.04-1.165].

velopment [27]. It was demonstrated that knock-out mice lacking only a single VEGF allele died on the 10th embryonic day because of the severely impaired formation of blood vessels [28].

Foetal genetic predisposition toward preterm birth has been widely investigated. In line with previous data suggesting the role of foetal genetic background in the aetiology of preterm birth, we found that prevalence of a mutant VEGF+405C allele is significantly higher in LBW infants than in healthy, term neonates. It is conceivable that the functional polymorphism of VEGF may influence prematurity by the alteration of VEGF production. Watson *et al.* demonstrated that in the presence of the VEGF+405CC genotype, the peripheral blood mononuclear cells produced less VEGF than those with the GG genotype [13]. We found that carriers of the VEGF+405 C allele, who are predisposed to low VEGF-producing ability have increased susceptibility to prematurity.

Considering that VEGF is essential for embryonic development, we can hypothesize that the decreased ability to synthesise VEGF could be an important component among the multiple aetiological factors of prematurity. However, we tested only the infants' genotype, and not the maternal genotype. Since half of the alleles in the newborn originate from the mother, we can only speculate whether this association is attributable to the infants' genotype, or the result of the mother's genotype. For this reason, further studies including the father, as well as the mother and the newborn, should be done to determine the importance of the VEGF G+405C polymorphism to preterm birth.

We also found that the carrier state for the VEGF-2578A allele is associated with more frequent development of NEC. Similarly to polymorphism of VEGF+405, VEGF-2578 is also associated with the regulation of VEGF synthesis; greater VEGF production was described in the peripheral blood mononuclear cells in those with the CC

Table 2

Vascular endothelial growth factor (VEGF) genetic polymorphisms in perinatal complications. (RDS: respiratory distress syndrome, NEC: enterocolitis necrotisans, IVH: intraventricular haemorraghia, ARF: acute renal failure, BPD: bronchopulmonary dysplasia, PDA: persistent ductus arteriosus, ASD: atrial septal defect)

	VEGF C^{2578} A genotypes CC/CA/AA prevalence of A allele (%)		VEGF T^{460} C genotypes TT/TC/CC prevalence of C allele (%)		VEGF G^{+405} C genotypes GG/GC/CC prevalence of C allele (%)	
Perinatal complication	Complication present	Complication absent	Complication present	Complication absent	Complication present	Complication absent
RDS (n=60 out of 124)	14/33/13 48	16/3315 49	15/31/14 49	15/34/15 50	26/32/2 60	33/26/5 29
NEC (n=49 out of 125)	6/27/16 **59***	24/40/12 41	9/27/13 54	21/39/16 47	23/25/1 27	37/33/6 30
IVH (n=42 out of 125)	10/24/8 49	22/42/12 49	10/23/9 49	2043/20 50	19/21/2 30	41/37/5 28
ARF (n=41 out of 107)	10/27/**4*** * 43	19/28/19 50	11/237/ 45	17/32/17 50	21/18/2 28	30/32/4 30
BPD (n=23 out of 125)	3/15/5 55	28/52/22 47	6/13/4 46	23/53/26 50	13/9/1 25	47/48/7 30
PDA (n=47 out of 125)	10/24/13 52	20/43/15 46	8/25/14 56	22/41/15 46	25/20/2 26	35/37/6 30
ASD (n=13 out of 110)	4/6/3 46	23/53/21 49	4/7/2 42	22/52/23 50	6/7/0 27	46/44/7 30

* Logistic regression analysis: p = 0.049, adjusted *odds ratio* [95% CI]: 2.77 [1.00-7.65]. ** Logistic regression analysis: p = 0.021, adjusted *odds ratio* [95% CI]: 0.2 [0.05-0.78].

genotype than those with AA genotype [14]. Our findings indicate that the carrier state for the VEGF-2578 mutant allele, which predisposes to low VEGF production, may enhance the susceptibility to NEC. The release of vasoconstrictor agents and proinflammatory cytokines in the small intestine are central processes in the pathogenesis of NEC [29]. Several lines of evidence support the notion that VEGF is involved in the regulation of the vasoconstriction-vasodilatation equilibrium and it also exerts anti-inflammatory effects [5, 30]. Although there are no data about the role of VEGF in the pathogenesis of NEC, we can speculate that these effects of VEGF may account for the association between the carrier state for the VEGF-2578 mutant allele, which predisposes to low VEGF production and the increased predisposition to NEC.

Interestingly, among the LBW infants with ARF, there were significantly fewer carriers of the VEGF-2578 AA genotype, which predisposes to low VEGF synthesis, than in the corresponding control group. It is known that VEGF is implicated in the pathogenesis of acute renal failure. It was demonstrated that VEGF synthesis is up-regulated in ARF induced by ischemia-reperfusion injury [31]. VEGF has an important role in maintaining the integrity of the glomerular filtration barrier, as inhibition of VEGF activity may lead to proteinuria [32]. It was observed that exogenous VEGF administration promotes endothelial cell proliferation and stabilizes renal function in a remnant kidney model [33]. Our findings may suggest that the carrier state of the VEGF-2578 AA genotype predisposing to low VEGF production has a possible protective effect in the development of ARF.

In the present study, we also investigated the association between VEGF SNPs and the risk of congenital heart disease. Interestingly, in contrast to our previous study [17] we did not find an association between the carrier state for VEGF G+405C and VEGF C-2578A and the risk of PDA or ASD.

A possible explanation for the different results could be the different populations investigated in the studies. In the earlier study, we enrolled a larger population of children who suffered from congenital heart defects (n = 102) as opposed to the present study where a smaller LBW population (n = 60) was investigated. Moreover, in our previous study we enrolled children between the ages of 2 and 10 years as opposed to the present study where we investigated a population of preterm infants.

However, when evaluating the association between the VEGF genotype and perinatal complications, it should be taken in account that the subgroups studied were small.

It is also conceivable that the observed associations between VEGF G+405C and VEGF C-2578A, preterm birth and complications such as NEC and ARF are not directly related to VEGF production capacity. The VEGF gene is located near to other genes (e.g. heat shock protein 70 [34], TNF-alpha [35], on chromosome 6 and which are possibly implicated in preterm birth). Therefore, it is possible that the association between the SNPs investigated and preterm birth and its complications are the result of linkage disequilibrium with other gene mutations.

In conclusion, we observed a higher prevalence of the VEGF +405C allele in LBW infants than in healthy, term neonates suggesting that the carrier state of this allele is a risk factor for preterm birth. Furthermore, we found an association between the carrier state for the VEGF-2578A

allele and the development of NEC in LBW infants. Interestingly, our study also suggests that the VEGF-2578AA genotype may have a protective role against ARF in LBW infants. These findings suggest that testing for VEGF SNPs would provide valuable information for the risk assessment of preterm birth and perinatal complications. However, because of the relatively small numbers in our study, the clinical significance of our results may be questioned, therefore it would be reasonable to extend the investigation using a larger population to determine VEGF levels.

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