Endothelial nitric oxide synthase gene polymorphism in women with idiopathic recurrent miscarriage

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BACKGROUND: Lack of endothelium-derived nitric oxide is associated with vasospasm and vascular infarction. We investigated the relationship between idiopathic recurrent miscarriage and a polymorphism of the gene encoding endothelial nitric oxide synthase (NOS3). METHOD: In a prospective case–control study, 105 women with idiopathic recurrent miscarriage and 91 healthy controls were investigated. We used the polymerase chain reaction to identify the different alleles of a 27 base pair tandem repeat polymorphism in intron 4 of the NOS3 gene. RESULTS: The wild type B allele was identified on 329 out of 392 chromosomes (frequency 0.84). The polymorphic A allele was present on 63 chromosomes (frequency 0.16). The genotype frequencies were as follows: 68% (B/B), 31% (A/B) and 5% (A/A). The distribution of genotype frequencies was significantly different between the study and control groups for allele A/B heterozygotes (NOS3A/B) (36.7 versus 23.8%, P = 0.03, OR 1.6, 95% CI 1.1–3.8). Only one individual was homozygous for the A allele (NOS3A/A). She was in the study group. Between women with primary and secondary recurrent miscarriages, no statistically significant difference between the distribution of NOS3A/B genotypes (28 versus 34%) was observed. CONCLUSIONS: These data support a role for the NOS3 gene as a genetic determinant of the risk of idiopathic recurrent miscarriage.

Key words: endothelial nitric oxide synthase/idiopathic recurrent miscarriage/nitric oxide/polymorphism/risk factor

Introduction

Clinically recognized pregnancies will end in a miscarriage in 15–20% of cases (Anonymous, 1995). Studies indicate that the majority of biochemical pregnancies, i.e. subclinical pregnancies with a positive serum or urine beta-human chorionic gonadotrophin (βHCG), are aborted spontaneously (Boklage et al., 1990). Clinical miscarriage, however, usually remains a single event in a woman’s reproductive life. Recurrent miscarriage, defined as three or more consecutive pregnancy losses before 20 weeks gestation, affects 0.5–2% of women (Wilcox et al., 1988). Whether recurrent miscarriage represents the common endpoint of independent etiologic factors or a distinct pathophysiological entity, is unknown. A wide variety of associated factors have been identified, among them uterine anomalies (Tho et al., 1979), luteal phase defect (Daya et al., 1989), hyperprolactinaemia (Kutteh et al., 1999), hyperandrogenaemia (Okon et al., 1996), hyperhomocysteinemia (Wouters et al., 1993), genital infections (Withkin and Ledger, 1992), and maternal/paternal balanced translocations (Husslein et al., 1982). Autoimmune dysfunctions, e.g. antiphospholipid syndrome, thyroid autoantibodies, and anti-single strand DNA autoantibodies, are found in 5–10% of affected women (Anonymous, 1995; Kutteh et al., 1996).

Association studies have been used to help understand the contribution of single genes towards the development of recurrent miscarriage. A role for the HLA system (Takakuwa et al., 1999), the pathway of folic acid metabolism (Ray and Laskin, 1999), and the blood clotting cascade have been described (Souza et al., 1999). Targeted mutations in mice have also been used to define the contribution of specific genes to the pathophysiology of miscarriage. This technology has affirmed a potential role for the gene encoding angiotensinogen in the aetiology of miscarriage (Tempfer et al., 2000a).

Nitric oxide (NO), released during the conversion of l-arginine to l-citrulline by nitric oxide synthase (NOS), is known to mediate vascular smooth muscle relaxation. NO has been implicated in the development of endothelial damage, hypertension, coronary spasm, and myocardial infarction (Nakayama et al., 1999; Brsic et al., 2000; Orange et al., 2000). We recently identified a genetic association between the gene encoding endothelial NOS (eNOS) and pre-eclampsia in an animal model (Gregg et al., 1999). In humans, NO has been considered a candidate molecule in the development of pre-eclampsia (Arngrimsson et al., 1987).

Endothelial NO synthase (eNOS) is expressed in terminal villous vessels and in the syncytiotrophoblast of pregnant...
women (Rossmanith et al., 1999). During the first trimester, placental production of HCG is modulated by eNOS expression and subsequent NO release of cyto- and syncytiotrophoblast cells (Sanyal et al., 2000). In mice, lipopolysaccharide (LPS)-induced abortion is mediated by placental NO production (Haddad et al., 1995) and pharmacologic inhibition of NO release by aminoguanidine successfully rescues LPS-induced abortion (Athanassakis et al., 1999).

Recently, a polymorphism in intron 4 of the gene encoding eNOS (NOS3) was shown to segregate with lower plasma NO metabolites in a nonpregnant Japanese population (Tsukada et al., 1998). The aim of our study was to determine if this polymorphism is associated with idiopathic recurrent miscarriage.

Materials and methods

Patients

The diagnosis of idiopathic recurrent miscarriage was based on a documented history of at least three spontaneous, consecutive miscarriages before 20 weeks gestation with the same partner. To avoid confounding by ethnicity, only white Caucasian women were included in the study and control groups. In addition, only women whose parents were of the same ethnicity were included in the study and control groups. One hundred and five women were included in the study group. These women underwent a standard diagnostic work-up to rule out a verifiable cause of the recurrent miscarriages. Diagnostic procedures included hysteroscopy, paternal and maternal karyotype, cervical cultures for chlamydia, ureaplasma, and mycoplasma, a comprehensive hormonal status, and evaluation of antiphospholipid syndrome with IgM and IgG anticardiolipin antibody assessment and lupus anticoagulant testing. Among these women, primary recurrent miscarriage was defined as no history of a pregnancy carried beyond 20 weeks gestation. Secondary recurrent miscarriage was defined as a history of at least one pregnancy carried beyond 20 weeks gestation. The control group consisted of 91 women with at least two live births and no history of miscarriage. All control women were postmenopausal to exclude the possibility of future miscarriages after inclusion in the study. Written informed consent was obtained from participating women.

Genotyping

DNA was obtained from blood using the QIAGEN System (QIAamp DANN Blood Midi Kit; Hilden, Germany). The extracted DNA was stored at 4°C until analysed. A genomic DNA fragment was amplified by the polymerase chain reaction (PCR) to determine the NOS3 genotype. Oligonucleotide primers flanking the 27-base pair repeat region in intron 4 of NOS3 were used. The forward primer was 5’-AGGCCCTATGCTAGTGCCTTT-3’. This primer was located at position 5111–5130 base pairs of the genomic sequence of NOS3. The reverse primer sequence was 5’-TCTCTTAGTGCTG-TGGTCAC-3’. The polymorphic allele (allele A) generated 0.84). The A allele was present on 63 chromosomes (frequency 31% (A/B) and 0.03 after inclusion in the study. Written informed consent was obtained from participating women.

Statistical analysis

Differences in the frequencies of the NOS3 alleles in the study and control groups were analysed by use of Fisher’s exact test. The odds ratio (OR) was used as a measure of the strength of the association between allele frequencies and recurrent idiopathic miscarriage. All P values were two-tailed and 95% confidence intervals (CI) were calculated. A P value < 0.05 was considered statistically significant.

Results

Characteristics of women with idiopathic recurrent miscarriage are shown in Table I.

The NOS3 genotype of 196 women was determined. The B allele was identified on 329 out of 392 chromosomes (frequency 84%). The A allele was present on 63 chromosomes (frequency 16%). These allele distributions are comparable to those reported in a US-Hispanic female population (Tempfer et al., 1999). The genotype frequencies were as follows: 68% (B/B), 31% (A/B) and 0.5% (A/A).

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heterozygotes (NOS3\textsuperscript{A/B}) (36.7 versus 23.8%, \( P = 0.03 \), OR 1.6, 95% CI 1.1–3.8). Only one individual was homozygous for the A allele (NOS3\textsuperscript{A/A}). She was in the study group.

The allele frequencies of NOS3 in the study and control groups and the associated ORs are shown in Table II.

Between women with primary and secondary recurrent miscarriages, no statistically significant difference between the distribution of NOS3\textsuperscript{A/B} genotypes (28 versus 34%) was observed.

Discussion
This study demonstrates an intron 4 polymorphism of the NOS3 gene to be associated with recurrent idiopathic miscarriage. Our data indicate that heterozygous carriers of the NOS3 polymorphism have a 1.6-fold increased risk of recurrent miscarriage compared to a control population. This adds further evidence to the concept of a polygenetic aetiological background of women with recurrent idiopathic miscarriage.

NO is known to play a functional role in ovulation. Inhibition of NO synthesis has been demonstrated to alter regional ovarian blood flow, inhibit follicle atresia, and reduce ovulatory efficiency and oocyte maturation (Jablonska-Shariff, 1999). Expression of eNOS has also been demonstrated in the placenta (Rossmanith et al., 1999) and is believed to modulate placentation and placental hormone production (Sanyal et al., 2000). Recently, we demonstrated that a lack of the gene encoding eNOS negatively impacts early embryonic development and survival in a mouse model (Tempfer et al., 2000b). The association between a NOS3 polymorphism and recurrent miscarriage, as demonstrated by the present study, further supports a functional role for NO in early embryonic development.

The data presented in this study provide evidence for a relationship between idiopathic recurrent miscarriage and a gene whose gene product is known to influence vascular smooth muscle reactivity. Given the aetiological role of NO deficiency in hypertension, vasospasm, haemorrhage, and infarction, it is reasonable to speculate that carriers of a NOS3 polymorphism and subsequently reduced NO serum levels are at increased risk for impaired placental perfusion and infarction. Impaired oxygen and nutrient supply might compromise the development of the embryo/fetus and its ability to resist maternal alloimmune rejection mechanisms.

Our data indicate that the investigated NOS3 polymorphism confers a small but significantly increased risk of developing idiopathic recurrent miscarriage. However, this polymorphism is neither necessary nor sufficient for the development of this condition. With respect to different genotype-phenotype correlations, others have reported similar results (Williams et al., 1994).

We describe an association between an intron polymorphism and recurrent miscarriage. Mechanisms which might explain the relationship between an intron polymorphism and a variation in the phenotype include: (i) linkage disequilibrium with a mutation within the coding region or promoter region of NOS3, (ii) the possibility of a neighbouring gene, that contributes to the development of preeclampsia, (iii) alteration of an as yet undescribed enhancer/suppressor element, and (iv) altered splicing or processing of the primary transcript. Although a series of genotype–phenotype relationships have been described for the NOS3 intron 4 polymorphism, the exact mechanism remains unknown.

Ethnic variation needs to be considered in an evaluation of the genetic background of IRM. The manner in which ethnic variation can influence the interpretation of association studies has been clearly demonstrated (Hartl and Clark, 1997). Thus, we made efforts to reduce error in the interpretation of our results by only considering white Caucasian women. In addition, genetic admixture may be a confounding factor in allele association studies (Knowler et al. 1988). Thus, only women whose parents were of the same ethnicity were included in the study and control groups. Another concern relates to the selection of a proper control group. Various other studies investigating women with recurrent miscarriage used age-matched controls to compare genotype frequencies. This strategy does not rule out future abortions among control women (Foka et al., 2000). To avoid this possible bias, all control patients in our series were postmenopausal at the time of blood sampling.

It is a limitation of our study that we cannot specify at what level a lack of eNOS derived NO affects the risk of developing recurrent miscarriage. Impaired NO production may affect early pregnancy at the gonadal level, placental level, or at the level of the systemic vasculature. Further studies are necessary to clarify the pathophysiological consequences of a lack of endothelium-derived NO for embryonic development.

In summary, the data presented in this study add further evidence to the concept of endothelium-derived NO as a physiological mediator of early pregnancy. Identification of a link between idiopathic recurrent miscarriage and a specific variant of a gene involved in the regulation of placental function and vascular homeostasis allows further insight into the natural history of this syndrome and allows further characterization of susceptible women.

References


Endothelial nitric oxide synthase gene polymorphism and recurrent miscarriage


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