The size of a microsatellite polymorphism of the haem oxygenase 1 gene is associated with idiopathic recurrent miscarriage

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Endothelial damage, impaired microvascularization and immune maladaptation have been described as aetiological factors in recurrent miscarriages. We investigated the relationship between idiopathic recurrent miscarriage (IRM) and a (GT)n repeat microsatellite polymorphism of the gene encoding haem oxygenase 1 (HO-1), known to modulate immune functions such as T-helper (TH) cell function and to be associated with cardiovascular disease. We investigated 162 women with IRM and 129 healthy, post-menopausal controls. The length of the HO-1 (GT)n microsatellite was assessed by PCR and direct sequencing in all women. Results were correlated with clinical data. The distribution of genotypes was in Hardy-Weinberg equilibrium. The HO-1 (GT)n microsatellite repeat numbers ranged from 13 to 37, with (GT)23 and (GT)30 being the most common alleles in both groups. We compared alleles consisting of <27 GT repeats, termed class S (short) alleles and alleles consisting of >28 GT repeats, termed class L (long) alleles. Seventy per cent of women with IRM had an S allele either in heterozygous (L/S) or homozygous (S/S) form, compared to 56% of controls (P = 0.02; OR 0.54; 95% CI 0.32–0.90). With respect to S allele frequencies, we found no significant difference among women with IRM and controls (P = 0.3; odds ratio (OR) 1.23, 95% confidence interval (CI) 0.86–1.76). Comparing women with primary and secondary IRM, no difference with respect to the length of the HO-1 (GT)n microsatellite was ascertained. In summary, this is the first report on a microsatellite polymorphism risk factor associated with IRM in a relatively large Caucasian population.

Key words: idiopathic recurrent miscarriage/haem oxygenase 1/microsatellite/polymorphism risk factor

Introduction

Recurrent miscarriage, defined as three or more consecutive pregnancy losses before 20 weeks gestation, affects 0.5–1% of women (Li et al., 2002). Successful pregnancy is dependent on the adequate function of the uteroplacental vascular system. Murine studies indicate that lack of vasodilating nitric oxide (NO) is sufficient to induce abortion. In humans, a relationship between idiopathic recurrent miscarriage (IRM) and a polymorphism of the endothelial NO synthase (NOS3) gene has been established (Tempfer et al., 2001). Endothelial damage resulting in microspasms and thrombosis at the maternal–fetal interface may also induce abortion (Aubard et al., 2000) and conditions associated with thrombosis and endothelial damage, e.g. hyperhomocysteinaemia, have been recognized as risk factors for recurrent miscarriage (Wouters et al., 1993). A recent meta-analysis of >30 studies documents an increased risk of early and late spontaneous as well as recurrent miscarriage in women with the Factor V Leiden and the G20210A mutation of the prothrombin gene (Rey et al., 2003).

Haem oxygenase (HO) is the rate-limiting enzyme in haem degradation, leading to the generation of free iron, biliverdin and carbon monoxide (CO) (Maines, 1988). Among the three identified mammalian HO isoforms, HO-1 is a stress-responsive protein, implicated in antioxidant defence mechanisms and modulation of vascular tone. Specifically, CO produced as a byproduct of HO-mediated haem degradation has been shown to share many properties with nitric oxide. By activating the soluble guanylate cyclase and increasing intracellular cGMP in endothelial cells (Furchgott and Jothianandan, 1991), it inhibits platelet aggregation, decreases endothelin-1 as well as platelet-derived growth factor production, and causes smooth muscle relaxation (Brune and Ullrich, 1987; Morita and Kourembanas, 1995). Several studies demonstrated an anti-atherogenic effect of HO-1 in the cardiovascular system (Duckers et al., 2001; Ishikawa et al., 2001). Besides vascular factors, immunological and inflammatory phenomena are thought to play a critical role in the pathogenesis of IRM. Pro-inflammatory, T-helper cell type 1 (TH-1)-associated cytokines promote abortion in rodents and humans (Li et al., 2002). A lack of the physiological TH-1/TH-2 balance shift and elevated serum levels of proinflammatory cytokines have been found among women with IRM.
Materials and methods

Patients

The diagnosis of IRM was based on a documented history of at least three spontaneous, consecutive miscarriages before 20 weeks gestation with the same partner. To avoid confounding factors of ethnicity, only white Caucasian women were included in the study and control groups. To avoid confounding factors of genetic admixture, only women whose parents were of the same ethnicity were included in the study and control groups. In all, 162 women were included in the study group between May 1999 and January 2003. Local Institutional Review Board approval was obtained. All of these women underwent a standard diagnostic work-up at the University of Vienna School of Medicine to rule out a verifiable cause of the recurrent miscarriages prior to inclusion in the study. Diagnostic procedures included hysteroscopy, paternal and maternal karyotype, cervical cultures for chlamydia, ureaplasma and mycoplasma, a comprehensive hormonal status (including estradiol, progesterone, FSH, LH, prolactin, testosterone and thyroid hormones T3 and T4), and evaluation of antiphospholipid syndrome with IgM and IgG anticardiolipin antibody assessment and lupus anticoagulant testing. Among these women, primary IRM was defined as no history of a pregnancy carried beyond 20 weeks gestation. Secondary IRM was defined as a history of at least one pregnancy carried beyond 20 weeks gestation. The control group consisted of 129 women with at least one live birth and no history of miscarriage. All control women were post-menopausal to rule out possible future miscarriages after inclusion in the study. Written informed consent was obtained from participating women.

Genetic studies

Blood was drawn from the antecubital vein and DNA was extracted using the Puregene System (Gentra Systems, USA). DNA was stored at 4°C until analysed. The 5′-flanking region of the HO-1 gene containing the (GT)n repeat was amplified by PCR using the forward primer AGAGCCTGC-AGCTTCTCAGA and the reverse primer ACAAAAGCTGGCCATAGGAC. Amplifications were performed in a GeneAmp 9700 thermocycler (Perkin Elmer Cetus, USA) under the following cycling conditions: 10 min denaturation at 95°C followed by 40 cycles of 30 s at 95°C and 2 min at 61.6°C and a final extension step at 72°C for 10 min. The PCR products were subjected to electrophoresis on high resolution Spandex EL 400 S-50 Wide Mini Gels (Elchrom Scientific, Switzerland) for 2.5 h at 144 V and 50°C constant buffer temperature. After staining with SybrGreen (Molecular Probes, USA) for 15 min and destaining with the destaining solution provided by the gel manufacturer for 30 min, the bands were analysed on a FluoroChrom Imaging System (Alpha Innotech, USA). Samples were sequenced on a Perkin Elmer 310 automated DNA sequencer, and samples with 25 and 30 repeats were included as size markers in every electrophoresis run. Two independent observers blinded with regard to patient clinical data evaluated the dinucleotide repeat length.

Statistical analysis

A power calculation was done: for detection of a 33% difference (1/3) with a 0.80 power, we needed 288 recruited patients after Yates’ correction. Differences in the frequencies of the number of GT-repeats of HO-1 in the study and control groups were analysed by χ²-test. In the case of multiple comparisons, Bonferroni’s correction was used. Normality of distribution was assessed by Kolmogorov-Smirnov test. The odds ratio (OR) was used as a measure of the strength of the association between allele frequencies and IRM. All P-values were two-tailed and 95% confidence intervals (CI) were calculated. P < 0.05 was considered statistically significant.

Results

In all, 162 women with IRM and 129 controls have been included in this study. Characteristics of women with IRM are shown in Table I. The allele frequencies of the HO-1 (GT)n microsatellite in women with IRM and controls are shown in Table II. The repeat numbers ranged from 13 to 37, with (GT)23 and (GT)30 being the most common alleles in both groups. This bimodal distribution of the microsatellite length is in good agreement with previous reports (Morita and Kourembanas, 1995; Li et al., 2002). The distributions of HO-1 (GT)n microsatellite genotype frequencies among women with IRM and controls are shown in Table III. We compared alleles consisting of ≈27 GT repeats, termed class S (short) alleles, and alleles consisting of >28 GT repeats, termed class L (long) alleles. Seventy per cent of women with IRM had an S allele either in heterozygous (L/S) or homozygous (S/S) form, whereas 56% of controls had an S allele either in heterozygous or homozygous form. This difference was statistically significant [P = 0.02; odds ratio (OR) 0.54; 95% confidence interval (CI) 0.32–0.90].

With respect to S allele frequencies, we found no significant difference among women with IRM and controls [P = 0.3; OR 1.23, 95% CI 0.86–1.76]. These data are shown in Table III.
Table III. HO-1 (GT)_n microsatellite polymorphism: genotype frequencies among women with idiopathic recurrent miscarriage and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Women with IRM (n = 162)</th>
<th>Controls (n = 129)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>L/L</td>
<td>49 (30)</td>
<td>57 (44)</td>
<td>1.16 (0.39–3.42); P = 0.8</td>
</tr>
<tr>
<td>S/S</td>
<td>88 (55)</td>
<td>47 (37)</td>
<td></td>
</tr>
<tr>
<td>L/S + S/S</td>
<td>25 (15)</td>
<td>25 (19)</td>
<td></td>
</tr>
<tr>
<td>L/S</td>
<td>74 (45)</td>
<td>82 (63)</td>
<td>2.07 (1.27–3.37); P = 0.003</td>
</tr>
<tr>
<td>S/S</td>
<td>113 (70)</td>
<td>72 (56)</td>
<td>0.54 (0.32–0.90); P = 0.02</td>
</tr>
</tbody>
</table>

^2-test.
IRM = idiopathic recurrent miscarriage; OR = odds ratio; CI = confidence interval.

Comparing women with primary and secondary IRM, no difference with respect to the length of the HO-1 (GT)_n microsatellite was ascertained.

Discussion

Several lines of evidence support an important role of the HO pathway in placental function and the maintenance of pregnancy. Induction of placental HO-1 has been shown to protect against TNF-alpha-induced cytotoxicity and to promote vascular relaxation (Ahmed et al., 2000). In agreement with the growth restriction observed in the pathogenesis of pregnancy loss (American College of Obstetricians and Gynecologists, 2001). The influence of the HO-1 (GT)_n microsatellite polymorphism on clinical phenomena such as abortion may not be only due to increased vascular stress, but also due to direct effects in the chorio-deciduala and trophoblast such as local immune protection. Besides these observations it is notable that the HO-1 expression is not only affected by the examined polymorphism length: other modifier genes and non-genetic confounders, such as vitamin E levels, known to functionally inhibit HO-1 messenger-RNA, influence the expression of HO-1 (Jenkins et al., 2001).

Finally, a fetal genetic contribution to the aetiology of IRM may provide an explanation for our finding of an association between short HO-1 (GT)_n repeats and IRM. Familial clustering of IRM has been described, suggesting an inherited component for this condition (Mowbray et al., 1991). Thus, increased inflammation and endothelial damage in families with IRM may induce a positive selection towards protective gene variants in the offspring. Subsequently, protective gene variants, such as short HO-1 (GT)_n repeat, may be over-represented among women in these families.

In summary, this is the first report of an HO-1 (GT)_n microsatellite polymorphism among women with IRM, demonstrating that the investigated polymorphism is associated with IRM in a large Caucasian population. The identification of a link between IRM and a specific variant of a gene involved in the regulation of vascular tone as well as maternal proinflammatory immune response allows further insight into the natural history of IRM and may contribute to a better characterization of susceptible women.

References


Yoshiki et al., 2000). In contrast to other organs, HO-1 was absent from the intima and media of placental vessels. Therefore, the influence of the HO-1 (GT)_n microsatellite polymorphism on clinical phenomena such as abortion may not be only due to increased vascular stress, but also due to direct effects in the chorio-deciduala and trophoblast such as local immune protection.

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