A polymorphism of the interleukin-6 gene promoter and idiopathic recurrent miscarriage

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BACKGROUND: Cytokines have been described to play a major role in the pathogenesis of idiopathic recurrent miscarriage (IRM). We investigated the association between IRM and a polymorphism of the interleukin-6 (IL-6) gene and IL-6 serum levels. METHODS: In a prospective case–control study, we studied 161 women with IRM and 124 healthy controls. Peripheral venous puncture, DNA extraction and PCR were employed to genotype women for the presence of a polymorphism at position −174 in the promoter region of IL-6. Serum IL-6 levels were assessed by a commercially available ELISA. RESULTS: Allele frequencies among women with IRM and controls were 63.4 and 58.1% respectively for allele G (wild type), and 36.6 and 41.9% respectively for allele C (mutant). No association between allele C and the occurrence of IRM was found (odds ratio 0.8; 95% confidence interval = 0.57–1.12; P = NS). IL-6 serum levels were not significantly different between genotypes and between the study and control groups. CONCLUSIONS: This is the first report on an IL-6 polymorphism in IRM. Although known to alter IL-6 expression, the IL-6 polymorphism investigated was not associated with IRM and alterations in IL-6 serum levels in a Middle-European Caucasian population.

Key words: cytokines/idiopathic recurrent miscarriage/interleukin-6/polymorphism/risk factor

Introduction

Physiologically, the maternal immune system confronts the embryo/fetus with a host-defence reaction, based on the recognition of paternally derived fetal and placental antigens (Beer, 1983). To avoid rejection of the semi-allogenic embryo/fetus, the maternal immune response is selectively suppressed in physiological pregnancies (Wegmann et al., 1993). While TH-2 type immunity is believed to contribute to successful pregnancy, TH-1 type immunity has been shown to be associated with idiopathic recurrent miscarriage (IRM) (Wegmann et al., 1993; Hill et al., 1995; Jenkins et al., 2000).

Murine studies indicate that dominance of TH-1 type dependent cytokines, e.g. interleukin 1 (IL-1), interleukin 2 (IL-2), tumour necrosis factor (TNF)-α, and interferon (IFN)-γ, are incompatible with successful pregnancy, whereas a dominance of TH-2 cytokines, e.g. IL-4, IL-6 and IL-10 prevents fetal wastage (Wegmann et al., 1993). Reports of elevated concentrations of TH-1 cytokines and reduced concentrations of TH-2 cytokines, including IL-6, among women with IRM are in accordance with these animal models (Lim et al., 2000; Makhseed et al., 2000; Shaarawy and Nagui, 1997).

IL-6 is a multifunctional cytokine, produced by many different cell types, including immune cells, fibroblasts, endothelial cells, adipocytes and myocytes (Papanicolaou et al., 1998). Secretion of IL-6 leads to a stimulation of the hypothalamic–pituitary–adrenal axis during inflammatory processes (Mastorakos et al., 1993), promotes osteoclastogenesis and participates in the development of osteoporosis associated with estrogen withdrawal (Manolagas and Jilka, 1995). IL-6 is not constitutively expressed, but is highly inducible and produced in response to a number of inflammatory stimuli (Wilson et al., 2001).

IL-6 is generally considered to be a proinflammatory cytokine. IL-6 also has anti-inflammatory properties, as demonstrated in IL-6 gene knock-out mice (Xing et al., 1998). The role of IL-6 expression during pregnancy, as well as its predictive value for pregnancy outcome, is unclear. Animal studies found that an increase in IL-6 concentrations precedes uterine contractions, suggesting that IL-6 plays a role in the physiological mechanisms involved in the propagation of labour (Gravett et al., 1994). In human studies, IL-6 production has been described in the decidua during early pregnancy. Also, IL-6 has been shown to induce the release of hCG from trophoblasts, leading to a subsequent cascade of progesterone production, release of Th2 cytokines, e.g. IL-6, IL-4, and suppression of TH-1 cytokines (Siiteri et al., 1977; Nishino et al.,
This is compatible with an anti-inflammatory role for IL-6 in pregnancy. On the other hand, elevated levels of IL-6 and proinflammatory cytokines, e.g. IL-1, TNF-α, and IL-8 in the placenta, amniotic cells, and decidua have been demonstrated in pregnancies complicated by preterm premature rupture of the membranes (pPROM), intrauterine infection and prematurity (Romero et al., 1990; Fukuda et al., 2002).

The gene encoding IL-6 has been mapped to chromosomal region 7p21 (Bowcock et al., 1988). A polymorphism in the 5’flanking region of the IL-6 gene (at position −174) has been reported, where the transcriptional response to stimuli such as endotoxins and interleukin-1 has been found to be associated with different plasma IL-6 levels in healthy volunteers (Fishman et al., 1998). Of note, the mutant C allele was found to be associated with significantly lower levels of plasma IL-6, whereas the wild type G allele was associated with higher IL-6 serum levels. Furthermore, an individual’s IL-6 genotype may be relevant to other conditions, such as juvenile systemic onset arthritis (Fishman et al., 1998), development of Kaposi sarcoma (Foster et al., 2000) and atherosclerosis (Fernandez-Real et al., 2001). These clinical data suggest that carriage of the mutated IL-6 allele C confers protection against the development and course of inflammatory diseases.

In this study, we attempted to establish an association between the polymorphism at position −174 in the promoter region of IL-6, IL-6 serum levels and the occurrence of IRM in a Middle-European Caucasian population.

Materials and methods

Patients

This study was approved by the internal review board at the University of Vienna School of Medicine.

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. A total of 161 women were included in the study group. Each woman underwent a diagnostic work-up to rule out the recurrent miscarriages. Diagnostic procedures included hysteroscopy, paternal and maternal karyotyping, cervical cultures for chlamydia, ureaplasma, and mycoplasma, a comprehensive hormonal status, and evaluation of antiphospholipid antibodies included hysteroscopy, paternal and maternal karyotyping, cervical cultures for chlamydia, ureaplasma, and mycoplasma, a comprehensive hormonal status, and evaluation of antiphospholipid antibodies.

Immunoglobulin M and IgG anticardiolipin antibody assessment and lupus anticoagulant testing. None of the women included in the study group were pregnant at the time of blood sampling. 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 124 women with at least two live births and no history of miscarriage. All control women were postmenopausal, to rule out possible future miscarriages after inclusion in the study. Written informed consent was obtained from participating women. To avoid confounding by ethnicity, only white Middle-European Caucasian women were included in the study and control groups. To avoid confounding by genetic admixture, only women whose parents were of the same ethnicity were enrolled.

Genotyping

Blood was drawn from the antecubital vein. Serum samples were stored at −80°C in aliquots to avoid possible interference with assay results due to repeated freeze–thaw cycles. DNA was extracted using the QIA Gen System (QIAamp DNA Blood Midi Kit; Qiagen GmbH, Hilden, Germany). DNA was stored at 4°C until analysis. For the determination of the IL-6 promoter polymorphism, we used a recently developed mutagenic separated PCR assay (MS-PCR) following the general principle described previously (Rust et al., 1993). Briefly, allele specific primers that differ in length by 8–10 base pairs (bp) with single base mismatches at defined positions were used, to minimize cross-reactions of the PCR products. In every sample, one or two different products are generated depending on the genotype. PCR amplification was carried out in 50 μl volumes containing 1.5 IU of AmpliTaq Gold (Perkin Elmer Cetus, Norwalk, CT), 1.5 mmol/l MgCl2, 200 mmol/l each of dNTP (Amersham Parmacia Biotech, Uppsala, Sweden), 8 pmol IL-6-174 G forward primer (5′-TCCCCCTAGTGTGTGGCAGC-3′), 12 pmol IL-6-174C forward primer (5′-CTGCACATTTACCCCTAGTGTGGCAGC-3′), 10 pmol IL-6 reverse primer (5′-TGAGGTGGCGCCACGAGCC-3′) (all MWG Biotech, Ebersberg, Germany) and ~50 ng DNA. PCR conditions comprised an initial denaturing step at 95°C for 10 min, followed by 37 cycles of 95°C for 1 min, 58°C for 2 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. Using this PCR

Table I. Demographic data of women with idiopathic recurrent miscarriage and controls

<table>
<thead>
<tr>
<th></th>
<th>Women with idiopathic recurrent miscarriage</th>
<th>Controls (n = 124)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>161</td>
<td>124</td>
</tr>
<tr>
<td>Age (years)</td>
<td>32 (23–43)</td>
<td>56 (40–81)</td>
</tr>
<tr>
<td>Ethnic background</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central European/Austrian</td>
<td>110</td>
<td>103</td>
</tr>
<tr>
<td>Central European/Non-Austrian</td>
<td>51</td>
<td>21</td>
</tr>
<tr>
<td>Purity</td>
<td>0.3 (0–3)</td>
<td>2.3 (2–5)</td>
</tr>
<tr>
<td>No. of miscarriages</td>
<td>3.8 (3–9)</td>
<td>0</td>
</tr>
<tr>
<td>Menopausal status</td>
<td>0</td>
<td>124</td>
</tr>
</tbody>
</table>

Values are given as median values and ranges.

Table II. IL-6 G (-174)C polymorphism: genotype frequencies among women with idiopathic recurrent miscarriage and controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Women with IRM (n = 161)</th>
<th>Controls (n = 124)</th>
<th>Odds ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/C</td>
<td>23 (14.3)</td>
<td>23 (18.5)</td>
<td>~</td>
</tr>
<tr>
<td>C/G</td>
<td>72 (44.7)</td>
<td>58 (46.8)</td>
<td>C/C versus C/G* 0.80 (0.3–2.08)</td>
</tr>
<tr>
<td>G/G</td>
<td>66 (41.0)</td>
<td>43 (34.7)</td>
<td>C/C versus G/G* 0.59 (0.06–5.6)</td>
</tr>
<tr>
<td>G/G + G/G</td>
<td>138 (85.7)</td>
<td>101 (81.5)</td>
<td>C/C versus G/G+G*/ 0.7 (0.15–3.2)</td>
</tr>
</tbody>
</table>

IRM = idiopathic recurrent miscarriage; *not significantly different (P > 0.05).
strategy, the wild-type allele (allele G) generated a 94 bp band. The mutant allele (allele C) generated a 103 bp band. PCR products were resolved on a 3% agarose gel and stained with SYBR Green I (FMC; Bio Products Europe, Denmark). In each experiment, a known individual heterozygous for allele C was included as positive control to ensure amplification of both alleles. A reagent control without DNA served as negative control.

Serum concentrations of IL-6 were measured using the commercially available enzyme linked immunosorbent assay (Quantikine®; Human IL-6 Immunoassay; R&D Systems, Minneapolis, MN, USA). All serum IL-6 analyses were performed at the same time, in the same batch, and in duplicate according to manufacturer’s instructions. The intra- and interassay coefficients of variation (CV) were 2.0–4.2% and 3.8–6.4% respectively.

**Statistical analysis**

Differences in the frequencies of the IL-6 alleles in the study and control groups were analysed by χ²-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% CI were calculated. The distribution of IL-6 serum levels was skewed and data are given as medians (range). Comparisons between the groups were made using the non-parametric Mann–Whitney U-test. The distribution of IL-6 serum levels above detection limit between cases and controls was made by χ²-test. P-values <0.05 were considered statistically significant.

**Results**

A total of 161 women with IRM were examined (Table I). Median age at diagnosis of women with IRM was 32 years (range 23–43). The median numbers of miscarriages and live births were 3.8 (range 3–9) and 0.3 (range 0–3), respectively. Of the 161 women, 52% were primary aborters and 48% were secondary aborters. A total of 124 women were examined as controls. Median age at time of blood sampling was 56 years (range 40–81). The median number of live births was 2.3 (range 2–5).

<table>
<thead>
<tr>
<th>Allele</th>
<th>Women with IRM (n = 161)</th>
<th>Controls (n = 124)</th>
<th>Odds ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>118 (36.6)</td>
<td>104 (41.9)</td>
<td>–</td>
</tr>
<tr>
<td>G</td>
<td>204 (63.4)</td>
<td>144 (58.1)</td>
<td>0.8* (0.57–1.12)</td>
</tr>
</tbody>
</table>

IRM = idiopathic recurrent miscarriage; *not significantly different (P > 0.05).

**Discussion**

In this study, we attempted to establish an association between a polymorphism in the promoter region of the IL-6 gene, known to decrease IL-6 protein expression, and the occurrence of IRM. Our hypothesis to test the IL-6 gene as a candidate gene for IRM was based on existing evidence that immunological processes are involved in the pathogenesis of this condition (Hill et al., 1995; Shaarawy and Nagui, 1997; Jenkins et al., 2000).

There are now several well documented instances where nucleotide polymorphisms occur within the regulatory region of cytokine genes, and some of these are associated with an altered rate of gene expression (Danis et al., 1995; Wilson et al., 1997). In addition, women with IRM have been found to carry a polymorphic allele of the IL-1 receptor antagonist gene (IL-1RN*2) more often than women without a compromised reproductive history (Unfried et al., 2001).

Evidence for a dichotomous T-helper response to trophoblast has been proposed to mediate reproductive success and failure, respectively (Hill et al., 1995; Raghupathy et al., 1999).

Recently, von Wolff and colleagues found an abnormal expression of IL-6 and IL-1 beta mRNA in endometrium during the mid-secretory phase in women with IRM (von Wolff et al., 2000). Others (Makhseed et al., 2001) reported significantly higher serum concentrations of the Th2 cytokines IL-6 and IL-10 at normal delivery than in women with IRM. Furthermore, higher IL-6 levels were found in women with IRM who had a successful pregnancy as compared with women with IRM who had another abortion (Makhseed et al., 2000). Thus, the dominance of Th2 cytokines seems to be of importance in maintaining pregnancy.

Our study, however, falls short of determining a significant effect of the IL-6 genotype on IRM. Furthermore, we could not ascertain any correlation between IL-6 serum levels and non-pregnant women with a history of IRM.
During pregnancy, IL-6 serum levels are detectable and increase significantly at the time of delivery (Austgulen et al., 1994; Makhseed et al., 2000). In those patients with intra-amniotic infection, high intra-amniol IL-6 levels are detectable (Romero et al., 1990). Thus, based upon these and our findings, a model of the role of IL-6 during pregnancy can be proposed. In normal pregnancy, IL-6 may be regarded as part of an anti-inflammatory mechanism aimed at maintaining pregnancy. Based on our data, there seems to be no effect of IL-6 on IRM. At the time of acute inflammatory responses such as in intra-amniotic infection, IL-6 seems to act as an inducer of acute phase reactions and an important player in the elicitation of cellular immune responses.

In summary, this is the first report of a genetic variant of the IL-6 promoter gene among women with IRM. We could demonstrate that the polymorphism at position −174 in the promoter region of IL-6 is not associated with altered IL-6 serum levels and IRM in a Middle-European Caucasian population. Based on our data, IL-6 seems not to be a candidate gene for this condition. Furthermore, serum IL-6 levels in the non-pregnant state are not associated with a candidate gene for this condition. Furthermore, serum IL-6 levels in the non-pregnant state are not associated with a patient’s history of IRM.

References


