Toll-like receptor 4 polymorphisms and idiopathic chromosomally normal miscarriage

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BACKGROUND: Lipopolysaccharide (LPS or endotoxin) exposure resulting from microbial invasion of the endometrium disturbs the Th1/Th2 balance at the feto-maternal interface and has been linked to the risk of idiopathic miscarriage in a range of human and animal studies. Toll-like receptor 4 (TLR4) mediates LPS signalling, and the human TLR4 gene harbours two single-nucleotide polymorphisms (SNPs) known to reduce LPS responsiveness. We hypothesized that genetic variation altering TLR4 function may influence the risk of idiopathic pregnancy loss.

METHODS AND RESULTS: We examined fetal TLR4 genotypes in a case-control cohort of chromosomally normal miscarriages (n = 96) and healthy term newborns (n = 113). The allele frequencies of the Asp299Gly and Thr399Ile TLR4 SNPs were determined by quantitative PCR using DNA extracted from extraembryonic tissues and umbilical cord blood, respectively. TLR4 genotype frequencies were not significantly different between cases and controls.

CONCLUSIONS: There was no association between fetal TLR4 polymorphisms, Asp299Gly and Thr399Ile, known to blunt LPS responsiveness, and the risk of idiopathic, chromosomally normal miscarriage. Nevertheless, TLR4 or perhaps other LPS-binding chaperone molecules are biologically plausible candidate genes that may alter the risk of idiopathic miscarriage.

Key words: miscarriage/TLR4/polymorphism/innate immunity/lipopolysaccharide

Introduction

Miscarriage, a clinically detectable pregnancy that fails to progress, occurs in ~15% of all pregnancies (Zinaman et al., 1996). Genetic (chromosomal) abnormalities explain at least half of all miscarriages. Although anatomical, endocrine, immune, infective and thrombophilic conditions are other possible causes, most chromosomally normal miscarriages remain unexplained or idiopathic.

Cytokines influence all steps of reproduction, including the risk of miscarriage. Specifically, Th2 cytokines [e.g. interleukin (IL)-4, IL-10 and IL-13] are associated with pregnancy success, whereas Th1 cytokine responses [e.g. interferon (IFN)-γ, and tumour necrosis factor (TNF)-α] predominate in spontaneous miscarriage (Hill et al., 1995; Marzi et al., 1996; Piccinni et al., 1998; Raghupathy et al., 1999; Michimata et al., 2003). Although the complexity of the cytokine network at the feto-maternal interface has increased with the discovery of newer cytokines and improved understanding of the role of specific cellular subtypes (e.g. natural killer cells, dendritic cells and regulatory T cells), the evidence that a Th1 response in the decidua may lead to miscarriage remains substantial (Romero et al., 2004).

Toll-like receptors (TLRs) are a recently identified group of vertebrate receptors that play a central role in determining the Th1/Th2 balance of immune responses. The human TLR family consists of 10 receptors that orchestrate the innate immune response by linking pathogen recognition with immune cell activation (Takeda et al., 2003). Individual TLRs recognize a distinct, but limited, repertoire of conserved microbial products, and the best-characterized receptor-ligand pair is TLR4 and lipopolysaccharide (LPS or endotoxin). In most situations, TLR4 activation promotes the generation of a Th1-dominated immune response and inhibits Th2 cytokine production (Dabbagh and Lewis, 2003).

Of the 10 human TLRs, at least three lines of experimental evidence suggest that TLR4 activation may contribute to the risk of idiopathic miscarriage by disturbing the Th1/Th2 balance at the feto-maternal interface. First, functional TLR4 is expressed by the placenta (Holmlund et al., 2002; Abrahams et al., 2004, 2005 Kumazaki et al., 2004). Second, TLR4 activation triggers the secretion of a range of Th1 cytokines experimentally implicated in infertility and pregnancy failure, including TNF-α, IL-1, IL-6 and IL-8 (Chaouat et al., 2004). Finally, LPS (the ligand for TLR4) is a well-recognized cause of miscarriage in a range of mammalian species (Daels et al., 1987; Giri et al., 1990; Clark et al., 1993, 1999, 2003, 2004; Wegmann et al., 1993; Schlafer et al., 1994; Baines et al., 1996).
1996; Deb et al., 2004). In humans, where the experimental administration of LPS is not acceptable, there is a growing body of evidence implicating LPS with the risk of miscarriage. Bacterial infections of the maternal genital tract (e.g. bacterial vaginosis) are associated with both histologic endometritis due to ascending intrauterine infection/inflammation (Korn et al., 1995; Peipert et al., 1997) and early pregnancy loss (Hay et al., 1994; Ralph et al., 1999). Recently, direct experimental data linking LPS exposure and miscarriage emerged from a study of women undergoing IVF-embryo transfer in which no successful pregnancies occurred in women with an elevated menstrual effluent LPS level of >200 pg/ml (Kamiyama et al., 2004). The model emerging from these data is that women with microbial invasion of the endometrium (due to Gram-negative bacteria and other microorganisms) may develop a Th1-biased proinflammatory response in the endometrium. This Th1 inflammatory response can predispose to a range of adverse pregnancy outcomes including damage of the conceptus, implantation failure, spontaneous miscarriage and preterm delivery (Romero et al., 2004).

The human TLR4 gene harbours two important non-synonymous single-nucleotide polymorphisms (SNPs)—Asp299Gly (refSNP ID: rs4986790) and Thr399Ile (refSNP ID: rs4986791)—reported to reduce LPS responsiveness (Arbour et al., 2000). Asp299Gly and Thr399Ile frequently cosegregate, and the allelic frequency of these polymorphisms has been estimated at 3–11% of the general population (Kiechl et al., 2002; Boekholdt et al., 2003; Hessen et al., 2002; Van Rijn et al., 2004). The biological relevance of these TLR4 SNPs has been widely investigated; individuals carrying the variant alleles are at increased risk of Gram-negative infections (Agnese et al., 2002; Lorenz et al., 2002b; Smirnova et al., 2003) and premature birth (Lorenz et al., 2002a) but are protected from atherosclerosis (Kiechl et al., 2002; Ameziane et al., 2003; Balistrieri et al., 2004). Currently, there is very limited understanding of how genetic factors controlling the innate immune system influence the risk of miscarriage in otherwise healthy women. Given the established association between LPS exposure, Th1/Th2 genetic factors controlling the innate immune system influence the risk of miscarriage in otherwise healthy women. Given the established association between LPS exposure, Th1/Th2

**Materials and Methods**

**DNA samples**

A case-control association study was conducted with genomic DNA samples obtained from 96 chromosomally normal spontaneous miscarriages (cases) and 113 healthy term newborns (controls). The majority of cases were first trimester miscarriages occurring at 8–12 weeks’ gestation. DNA was isolated from: (i) chorionic villi or amnion samples that were obtained anonymously from products of conception submitted for chromosome analysis to the Embryopathology Laboratory at the BC Children’s & Women’s Hospital and (ii) umbilical cord blood of healthy term neonates. DNA collection was approved by the University of British Columbia’s Clinical Research Ethics Board.

Cases of chromosomally normal miscarriage were ascertained through screening cytogenetic records from karyotyped miscarriage specimens at BC Children’s & Women’s Hospital. The most common indication for karyotyping was a previous miscarriage. Other less-common indications included advanced maternal age, infertility, IVF/ICSI and abnormal conceptus. At this centre, all specimens in which there was culture failure or in which the karyotype was 46,XX were repeated by comparative genomic hybridization for verification and improved cytogenetic accuracy (Lomax et al., 2000).

**TLR4 genotyping by quantitative PCR**

Genotyping of TLR4 allelic variants, Asp299Gly (refSNP ID: rs4986790) and Thr399Ile (refSNP ID: rs4986791), was performed by quantitative PCR as previously described (Van Rijn et al., 2004). Briefly, probe pairs for each SNP had either FAM (Asp299 and Thr399) or VIC (299Gly and 399Ile) fluorescent labels on the 5’-end and non-fluorescent quenchers on the 3’-end. The alleles of the Asp299Gly or Thr399Ile SNPs were discriminated by measuring free FAM (492 nm excitation, 520 nm emission) and VIC (520 nm excitation, 550 nm emission) fluorescence in real-time during the PCR.

The region containing the Asp299Gly polymorphism was amplified using the following primer pair: forward 5’-TGAAGAATTCGATT- AGCATACTTGA-3’ and reverse 5’-TGTGGGAAACTGTC- CAAAATTTCA-3’. The Thr399Ile region was amplified using forward primer 5’-TGAAGTATTCAAAGGTGTCTGTTC-3’ and reverse primer 5’-AGGAATCTGAAACTCCTATTGTT-3’. Allele-specific probes were constructed by dual labelling with a fluorescent label and a non-fluorescent quencher conjugated to a minor groove binder (MGB). Probes to detect Asp299 (FAM) and 299Gly (VIC) were 5’-[FAM]-ACCCTGATGATAT-[MGB]-[quencher]-3’ and 5’-[VIC]-ACCTCGATTGATAT-[MGB]-[quencher]-3’, while probes to detect Thr399 (FAM) and Ile (VIC) were 5’-[FAM]-TATAGGCTGGTTGCC-[MGB]-[quencher]-3’ and 5’-[VIC]-TTAGGCTGGTCC-[MGB]-[quencher]-3’. PCR reactions (25 µl) were run in well 96-well optical reaction plates (Applied Biosystems) and consisted of genomic DNA (200 ng), SNP-specific primers (200 µM) and probes (200 µM), 2x TaqMan Universal PCR Master Mix (Applied Biosystems) and distilled H2O. The fluorescence was detected in real time during PCR thermal cycling using an ABI 7000 Sequence Detection System (Applied Biosystems).

**Statistical analysis**

A chi-square test was performed to determine whether there was an association between the allelic frequencies of the case and control groups, and $P < 0.05$ was considered significant.

**Results**

Genotyping of TLR4 allelic variants, Asp299Gly and Thr399Ile, was performed on 96 fetal DNA samples isolated from chromosomally normal miscarriages and 113 healthy term newborns (Table I). Asp299 and Thr399 alleles co-segregated in 94 of 96 (98%) case samples and in 111 of 113 (98%) control samples. Allele frequencies in both groups were found to be in Hardy–Weinberg equilibrium.

Corresponding allele frequencies in the cases of chromosomally normal miscarriage were 5.2% for both 299Gly and 399Ile. In the control group of healthy term neonates, the allele frequencies were 4.0% for both 299Gly and 399Ile. The ratios of homozygous and heterozygous subjects for both Asp299Gly
and Thr399Ile in the case and control groups were not significantly different when analysed by the chi-square test (P > 0.05).

### Discussion

We have shown no association between TLR4 polymorphisms reported to blunt LPS responsiveness and the risk of idiopathic, chromosomally normal miscarriage. Although our study was relatively small in size, the lack of even a subtle difference makes it unlikely that variation in fetal TLR4 genotype significantly alters the risk of idiopathic pregnancy loss.

Our result does not entirely rule out an important role for genetic variation in LPS responsiveness in determining the risk of idiopathic pregnancy loss. First, we were only able to examine TLR4 genotypes of the fetus, and it is possible that maternal TLR4 genotype is related to the risk of miscarriage. Our decision to examine the fetal genotype was based on the observation that the fetal 299Gly allele of TLR4 has been associated with increased risk of premature birth (Lorenz et al., 2002a). Second, the interaction between LPS and TLR4 also relies on a range of chaperone molecules including LPS-binding protein (LBP), CD14 and MD2. Therefore, either maternal or fetal variation in any of these molecules may influence gestational outcome. Indeed, others have examined the link between maternal CD14 promoter polymorphisms and the risk of idiopathic recurrent miscarriage but failed to detect any significant association (Karhukorpi et al., 2003).

It is also important to recognize that the potential functional relevance of these TLR4 SNPs has come under question. The initial description of the TLR4 SNPs demonstrated that individuals heterozygous for these polymorphisms have reduced airway responsiveness to inhaled LPS in vivo, and primary airway epithelial cells derived from heterozygous donors have a diminished LPS response in vitro (Arbour et al., 2000). However, a number of recent studies have failed to reproduce these initial findings. Peripheral blood cells derived from individuals heterozygous and homozygous for the Asp299Gly and Thr399Ile TLR4 variants have been shown to respond to LPS in a fashion indistinguishable from wild-type controls (Erridge et al., 2003; von Aulock et al., 2003; van der Graaf et al., 2005). Hence, our inability to demonstrate an association between TLR4 polymorphisms and the risk of idiopathic, chromosomally normal miscarriage may reflect the fact that the Asp299Gly and Thr399Ile TLR4 SNPs do not modify the capacity of cells to respond to TLR4 agonists.

In conclusion, our findings suggest that the risk of idiopathic miscarriage is not influenced by the fetal Asp299Gly and Thr399Ile TLR4 genotype. Nevertheless, the genes controlling LPS responsiveness are biologically plausible candidate genes that may contribute to the risk of unexplained pregnancy loss. Further studies will be beneficial in defining how maternal and fetal genetic variations controlling the innate immune response contribute to idiopathic miscarriage.

### Table 1. Toll-like receptor 4 single-nucleotide polymorphisms allele frequencies

<table>
<thead>
<tr>
<th>Allele</th>
<th>Healthy term neonates</th>
<th>Chromosomally normal miscarriage</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp299Gly</td>
<td>G</td>
<td></td>
<td>G</td>
</tr>
<tr>
<td>Genotype</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Asp/Asp</td>
<td>217 (96.0)</td>
<td>182 (94.8)</td>
<td>0.54</td>
</tr>
<tr>
<td>Asp/Gly</td>
<td>9 (4.0)</td>
<td>10 (5.2)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>226</td>
<td>192</td>
<td></td>
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</tbody>
</table>

Allele frequencies of the TLR4 SNPs Asp299Gly and Thr399Ile determined by quantitative PCR.

### References


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