Toll receptor 4 Asp299Gly polymorphism and its association with preterm birth and premature rupture of membranes in a South American population

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Preterm birth (PTB) is a worldwide health problem and remains the leading cause of perinatal morbidity and mortality. Systemic and local intrauterine infections have been implicated in the pathogenesis of preterm labor and delivery. Common pathways between PTB, premature rupture of ovular membranes (PROM) and altered molecular routes of inflammation have been proposed. There is evidence to support a genetic component in these conditions. Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is thought to play a key role in eliciting an inflammatory response. LPS is recognized by proteins of the innate immune system, including Toll-like receptor 4 (TLR4). Individuals from some European countries carrying the variant alleles resulting in an amino acid substitution (Asp299Gly) are at increased risk of Gram-negative infections and premature birth. The objective of this study was to determine if preterm newborns have different allele frequency of the Asp299Gly TLR4 variant from healthy term neonates in Uruguay. The impact of PROM was also examined. There was an increase in the risk for fetuses carrying the Asp299Gly substitution in TLR4 of being severely premature (<33 weeks) and to present PROM at the same time.

Keywords: TLR4 polymorphism; preterm birth; premature rupture of membranes; genetic association; South American population

Introduction

Preterm birth (PTB), defined as the delivery of a fetus that has not completed 37 weeks of gestation, is a worldwide health problem. Preterm delivery remains the leading cause of perinatal morbidity and mortality (Esplin, 2006). Prematurity results in a 40-fold increase in neonatal mortality (Rush et al., 1976; Goldenberg and Rouse, 1998; Goldenberg et al., 1998). Among surviving infants, PTB is implicated in approximately half of all pediatric neurodevelopmental disabilities, including cerebral palsy (Goldenberg and Rouse, 1998), long-term morbidity and high healthcare costs (Ananth and Vintzileos, 2006). Unfortunately, the rate of PTB continues to rise according to recent incidence reports, and is currently 12–13% in the USA (Goldenberg et al., 2001; Ananth et al., 2006; Ananth, 2007). In South America, more than 10% of newborns are preterm, and the incidence is between 8% and 10% in Uruguay (http://www.msp.gub.uy/subcategoria_4_1_1.html). The rising incidence of PTB is linked to an increase of iatrogenic termination of pregnancy associated with severe pre-eclampsia, intrauterine growth restriction, severe maternal health conditions and others. However, an increase of spontaneous preterm delivery has also been reported (Ananth et al., 2006).

Despite the impact of spontaneous PTB on current obstetric practice, our understanding of this complication is still not clear. Spontaneous PTB probably has different underlying causes (Norwitz et al., 1999). Recognized etiologies of PTB such as maternal and fetal stress, inflammation, infection and hemorrhage or placental abruption ultimately converge in a final common pathway that leads to organized uterine contractions, rupture of membranes and cervical changes followed by preterm delivery (Lettieri et al., 1993). Systemic and local intrauterine infections have been implicated in the pathogenesis of preterm labor and delivery (Romero et al., 1987, 1988; Romero and Mazor, 1988). Indeed, either local or systemic exposure to microbial products leads to PTB in several animal models (Elowitz and Mrinalini, 2004). Intrauterine infection has been implicated in the etiology of preterm premature rupture of ovular membranes (PPROM), which accounts for 30–40% of all preterm deliveries (Parry and Strauss, 1998). Common pathways between PTB, PPROM and altered molecular routes of inflammation have been proposed. This common pathway could occur without even the presence of chorioamnionitis or clinical infection (Romero et al., 2006).

The documented increased risks in patients with a personal or family history of PTB and the ethnic disparities in the incidence of PTB suggest a genetic component in this condition (Varner and Esplin, 2005). In this way, preterm delivery appears to be a complex mechanism determined by both genetics and environmental factors.

Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is thought to play a key role in eliciting an inflammatory response including the activation of the immune cells and the release of enzymes involved in remodeling of the extracellular matrix leading to PPROM and PTB (Parry and Strauss, 1998). LPS is recognized by proteins of the innate immune system, including...
Toll-like receptor 4 (TLR4) (Medzhitov et al., 1997). Hyporesponsiveness to an LPS challenge has been associated with a TLR4 variant resulting in an amino acid substitution (Asp299Gly) lying between leucine-rich repeats (Arbour et al., 2000).

The biological relevance of this TLR4 single nucleotide polymorphisms (SNPs) has been widely investigated; individuals carrying the variant alleles are at increased risk of Gram-negative infections (Agneze et al., 2002; Lorenz et al., 2002b), and premature birth (Lorenz et al., 2002a) but not for PPROM in African Americans (Ferrand et al., 2002a). The same variant protects individuals from atherosclerosis (Kiechl et al., 2002; Ameziane et al., 2003).

In this research, we first determined the allele frequency of the Asp299Gly TLR4 gene polymorphism in term Uruguayan neonates. Then, we examined TLR4 genotypes in a cohort of preterm neonates. We considered PROM as an inflammatory co-factor of PTB and studied the impact of carrying this polymorphism in newborns that had PROM.

The discovery of genetic factors involved on PTB may lead to medical breakthroughs and reduction therefore in spontaneous PTB, neonatal morbidity and mortality. Understanding causes of PTB is especially important in underdeveloped countries. Complications of PTB are associated with increase of medical expenses, representing billions of dollars of direct costs and unrealized potential each year in developed countries (Esplin, 2006) and unaffordable for poor countries.

Materials and Methods

Subjects

Subjects in this study were offspring of women receiving obstetric care at the Pereira Rossel Hospital, Montevideo, Uruguay. The study protocol was approved by the School of Medicine Ethics Committee of the Republic University, Uruguay. Subjects were recruited sequentially between May 2003 and May 2008. Informed written consent was obtained from mothers prior to collection of biological material, including specimens for extraction of DNA.

Cases (n=226) were neonates from pregnancies complicated by spontaneous PTB. Two subgroups were distinguished; neonates born between 33 and 36 weeks were considered as moderate PTB (n=118) and neonates of gestational age (GA) < 33 weeks were included in a group of severe PTB (n=108). Control subjects (n=250) were neonates delivered at term. Subjects with PROM were detected in all categories. Patients with multiple gestations and fetal anomalies were excluded.

Analysis of TLR4 alleles

DNA was extracted either from umbilical cords or from newborn cheek swabs by conventional methods (Ferrand et al., 2002b). To detect TLR4 896 A>G non-synonymous polymorphism, we used the PCR amplification strategy previously described (Lorenz et al., 2001; Ferrand et al., 2002a), employing mismatch primers designed to detect the wild-type and variant TLR4 alleles based on the presence of a restriction site in the variant allele. The following primers were used: forward 5'-GATTCAGATATCTTGGATCTTCCATG-3' and reverse 5'-GATTAGACTTCTGAAAAAGCATTCCCAC-3'. The underlined base in the forward primer indicates the nucleotide altered to create an NcoI restriction site in the presence of the polymorphism. PCR was carried out using PureTaq™ Ready-To-Go™ PCR beads (Amersham Biosciences). After initial denaturation at 94°C for 5 min, PCR was performed for 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension for 30 s at 72°C, after which a 20 µl sample was digested with NcoI (New England Biolabs) and fractionated on a 3% agarose gel.

Statistical analysis

Cases and controls were compared on demographic factors using ANOVA followed by the Tukey test. Comparisons of proportions of allele frequencies and the Hardy–Weinberg equilibrium test were performed using the $\chi^2$ test or Fisher's exact test (Guo and Thompson, 1992).

The association between TLR4 genotypes and PROM related to GA was examined by logistic regression analysis. The presence of differential effects of TLR4 genotypes on the risk of PROM was explored by the inclusion of interaction or conditional terms. The analysis was performed with the Epi Info 2000 software (Center for Disease Control and Prevention, Atlanta, GA, USA). Probability values of 0.05 or less were considered significant.

Results

To determine if there was an association between PTB and the hyporesponsive TLR4 allele, we performed a case–control study in which we genotyped fetal DNA from cases (PTB < 37 weeks) and controls. Since severe PTB showed particular susceptibility to inflammation, two groups of PTB were distinguished: moderate and severe newborns. The demographic characteristics of our study population are presented in Table I. Mothers were similar in age, parity and previous reproductive history. As expected, there were significant differences in birthweight of newborns and percentage of PROM.

Regarding the TLR4 polymorphism, we found that all carriers of the Asp299Gly allele were heterozygotes.

The TLR4 Asp299Gly allele frequency in the Uruguayan population shows no strong differences from other studies (Table II).

There was no significant difference in the frequencies of the TLR4 polymorphism among cases and controls (28 carriers in a total of 250 newborns of 37 weeks or more versus 25 carriers in 226 PTB; odds ratio 0.99; 95% confidence interval 0.557–1.748).

Regarding the combination of both effects, GA and PROM, allele frequency of the Asp299Gly variant was higher only in the severe PTB group with PROM versus without PROM. This difference was statistically significant (Table III).

The presence of TLR4 polymorphism was not associated with GA. When we analyzed PROM related to GA conditional on TLR4, a significant association in the severe preterm group was found (Table IV).

Table I. Clinical and demographic characteristics.

<table>
<thead>
<tr>
<th>n</th>
<th>≥37 weeks</th>
<th>33–36 weeks</th>
<th>&lt;33 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)*</td>
<td>250</td>
<td>118</td>
<td>108</td>
</tr>
<tr>
<td>Parity (% of nulliparous mothers)</td>
<td>26</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Birthweight (g)</td>
<td>3184 ± 30</td>
<td>2226 ± 32*</td>
<td>1414 ± 41*</td>
</tr>
<tr>
<td>Poor obstetric history (%)b</td>
<td>8</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>PROM (%)</td>
<td>20</td>
<td>40*</td>
<td>38*</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± SEM.  
*Previous preterm birth, low birthweight, stillbirth or recurrent miscarriage.  
*P < 0.05 versus term newborn.  
*P < 0.05 versus preterm 33–36 weeks.

Table II. TLR 4 Asp299Gly allele frequencies in different populations.

<table>
<thead>
<tr>
<th></th>
<th>Asp299Gly</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finland (Lorenz et al., 2002a)</td>
<td>0.083</td>
<td>351</td>
</tr>
<tr>
<td>USA (Arbour et al., 2000)</td>
<td>0.066</td>
<td>155</td>
</tr>
<tr>
<td>Mexico (Garza-Gonzalez et al., 2007)</td>
<td>0.077</td>
<td>259</td>
</tr>
<tr>
<td>China (Gao et al., 2007)</td>
<td>0.056</td>
<td>80</td>
</tr>
<tr>
<td>Portugal (Carvalho et al., 2007)</td>
<td>0.055</td>
<td>388</td>
</tr>
<tr>
<td>Africa (Mckenhaupt et al., 2006)</td>
<td>0.095</td>
<td>290</td>
</tr>
<tr>
<td>African American (Ferrand et al., 2002a)</td>
<td>0.066</td>
<td>218</td>
</tr>
<tr>
<td>Chile (Santos et al., 2006)</td>
<td>0.046</td>
<td>227</td>
</tr>
<tr>
<td>Spain (Santin et al., 2006)</td>
<td>0.08</td>
<td>269</td>
</tr>
<tr>
<td>Uruguay (present study)</td>
<td>0.05</td>
<td>250</td>
</tr>
</tbody>
</table>

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Logistic regression analysis showed evidence of an association between PROM and GA as it has been already described in Table I (Table IV).

The observed genotype distributions did not deviate from the Hardy–Weinberg equilibrium in any of the GA groups (Table III).

**Discussion**

We have detected an increase of the risk for fetuses carrying the Asp299Gly substitution in TLR4 of being severely premature (<33 weeks) and to present PROM at the same time. Heterozygous individuals showing an increased risk of presenting both effects represents a new finding regarding the action of TLR4.

In a Finish population, Lorenz et al. (2002a) showed that preterm singleton infants had a significantly higher carrier rate for the TLR4 Asp299Gly variant compared with term singleton infants. These data suggest an association of this polymorphism and PTB of singleton newborns. In agreement with Lorenz et al., we found that the Asp299Gly allele frequencies in severe preterm infants presenting PROM were higher than in the term infants. However, there is no significant association between the TLR4 Asp299Gly polymorphism and the risk of preterm delivery in our population when this complication was considered independently of membrane rupture.

In a recent study of more than 500 newborns, Krediet et al. found a significantly higher frequency of the TLR2 polymorphism Arg753Gln in premature neonates of <30 weeks gestation. Carriage of the variant TLR2 alleles potentially leads to aberrant innate immune responses, which may have contributed to severe PTB (Krediet et al., 2007). Interestingly, a tendency toward the latter association was also found in the Finnish study, but this did not reach statistical significance. To date, these are the only studies that have looked at the impact of different polymorphism on the inflammatory diseases in South American population which suggests the effect of admixture on this gene. The studies of some of these genetic variations require the presence of certain environmental stimuli to have any clinical significance. Macones et al. (2004) described a gene–environment interaction between the tumor necrosis factor (TNF) alpha polymorphism and bacterial vaginosis. Women found to have both the TNF alpha polymorphism and bacterial vaginosis were at synergistically increased risk of PTB when compared with women with only the TNF alpha polymorphism or bacterial vaginosis alone. We did not check for bacterial vaginosis in all of our patients, so we cannot rule out the possibility of the influence of this factor.

The variations of the TLR4 gene may impact in different ways according to the genetic background of the population and that could explain the differences between the Finish and Uruguayan populations. Ancestral origin differences in PTB have been well documented in the USA. African American women present twice the rate of PTB compared with European American women, even when confounding social and economic variables are controlled (Schieve and Handler, 1996). However, regarding TLR4 Asp299Gly polymorphism, there is no difference between these populations with respect to PPROM (Ferrand et al., 2002a). The prevalence of the polymorphism seems to be similar in all the populations studied so far (Table II). The data were collected from different sources and it is difficult to establish an accurate worldwide distribution, but it seems that African and European populations have the highest frequencies. Interestingly, the studies that found a relevance of this TLR4 SNP were carried out in populations with a high frequency of TLR4 Asp299Gly polymorphism as in a Finnish study (Lorenz et al., 2002a).

The structure of the population can explain the differences found in our study. Uruguayan population has been described fundamentally as of European origin. However, more recently, genetic admixture analysis demonstrated a Native American and African contribution to the Uruguayan population of 10.4% and 5.6%, respectively (Sanz et al., 1997; Hidalgo et al., 2005). The Uruguayan Asp299Gly observed frequency is less than that of Europeans or Africans and equal to the admixed Chilean population (Santos et al., 2006), which suggests the effect of admixture on this gene. The studies of the structure of different polymorphism on the inflammatory diseases in South American population have only recently started and sufficient data have yet to be collected and reported (Santos et al., 2006; Garza-Gonzalez et al., 2007). This is the first report concerning association between TLR4 and PTB or PPROM on a South American country.

TLR4 Asp299Gly allele frequencies are similar between the studied populations and none of them has an allele frequency higher than 10% (Table II). This is consistent with the idea that a mildly negative selection pressure is not sufficient to eliminate the allele (Smirnova et al., 2001) and that could explain the frequencies found in the worldwide populations. Modifications on the effect of TLR4 produced by TLR4 Asp299Gly polymorphism are not so deleterious by themselves, which means that selective evolutionary process has not been strong enough to eliminate the variant. Recently, an SNP in the promoter of the SERPINH1 gene was found. This variation of the SERPINH1 gene is found. Variation of the SERPINH1 gene is found.

**Table III.** TLR4 Asp299Gly allele frequencies in neonates delivered with or without PROM.

<table>
<thead>
<tr>
<th></th>
<th>Without PROM*</th>
<th>PROM*</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asp299Gly N</td>
<td>Asp299Gly N</td>
<td></td>
</tr>
<tr>
<td>≥37 weeks</td>
<td>0.050 200</td>
<td>0.080 50</td>
<td>0.89</td>
</tr>
<tr>
<td>33–36 weeks</td>
<td>0.029 70</td>
<td>0.042 48</td>
<td>1.00</td>
</tr>
<tr>
<td>&lt;33 weeks</td>
<td>0.045 67</td>
<td>0.134* 41</td>
<td>0.935</td>
</tr>
</tbody>
</table>

*Hardy–Weinberg test results for each GA group.
*P < 0.05 versus without PROM same GA.

**Table IV.** Logistic regression and ORs on TLR4 genotype and PROM of control samples versus 33–36 or <33 weeks.

<table>
<thead>
<tr>
<th></th>
<th>33–36 weeks</th>
<th>&lt;33 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>TLR4</td>
<td>0.546 (0.180–1.655)</td>
<td>0.285</td>
</tr>
<tr>
<td>PROM</td>
<td>2.857 (1.719–4.750)</td>
<td>0.000</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.875 (0.162–4.735)</td>
<td>0.877</td>
</tr>
<tr>
<td>PPROM conditional on TLR4</td>
<td>2.500 (0.500–12.511)</td>
<td>0.395</td>
</tr>
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gene reduces promoter function in amnion fibroblast cells and is strongly associated with risk of PPROM, the leading identifiable cause of PTB. This SNP is enriched in individuals of African ancestry (Wang et al., 2006). Once again, ethnic origin and the environment interact in order to determine the distribution of allele variants.

Recent evidence suggests that TLR4 SNPs 299 and 399 haplotypes are geographically clustered and this could be related to the susceptibility to infection, especially malaria, because it has a high prevalence in sub-Saharan Africa. There should be a beneficial effect of Asp299Gly in malaria that seems to override the negative effect in Gram-negative infections. The data provide an understanding of how our innate immune system has been molded by infectious pressures (Ferwerda et al., 2007). The high prevalence of Gram-negative infectious diseases in pregnancies in South American countries and Hispanic populations (Conde-Gonzalez et al., 1987; Gonzalez Pedraza et al., 1995; Gunn et al., 1995; Narcio et al., 1996) makes especially relevant the studies of the role of genes and polymorphisms in the susceptibility to infections.

If a multitude of rare variants (rather than restricted number of common variants) underlie the genetic susceptibility to these traits, we need more powerful strategies than association studies such as we performed here. Admixture mapping or linkage disequilibrium mapping strategies would be options that would help to uncover the effect of uncommon alleles (Reich and Patterson, 2005).

Ferrand et al. (2002a) detected no risk for newborns carrying the Asp299Gly variant of having preterm PROM. Interestingly, we have been able to detect an increased risk of PROM in fetuses carrying the Asp299Gly substitution only in severe PTB, a condition that was not considered separately in their report. Their research was carried out in an African American population so ethnic variations could explain different predispositions to inflammatory pathologies, underlying again the importance of population studies. Further investigations should be done where PROM and ethnic differences would be closely considered.

Our findings underline a role for genetic variation in LPS responsiveness in determining the risk of obstetric complications that leads to PTB only in the group of severe preterm. However, 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. Additional studies involving other polymorphisms of such genes as CD14, LBP and MD-2 may further substantiate the importance of host innate immunity genes as risk factors for pregnancy complications leading to premature birth and severe PTB (Hirschfeld et al., 2007).

Hence, our data show an association between TLR4 polymorphisms and the risk of PTB and PROM in the group of severe preterm suggesting differences of susceptibility to the inflammatory process between preterm neonates. However, in order to better understand the role of TLR4 on these obstetrical complications, novel strategies need to be developed to further uncover the effects of environmental factors, other genes and genetic backgrounds.

Funding
Funding for this work was provided by Fogarty International Center ‘National Institutes of Health’ grant RO1TW006223.

Acknowledgement
We acknowledge Robert O’Neill, English Language Centre, Hove, UK, for his careful review of the manuscript language and Maria de los Angeles Michel, for her assistance with medical records.

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Submitted on December 27, 2007; resubmitted on August 8, 2008; accepted on August 12, 2008.