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Tumor Necrosis Factor-α Allele Lymphotoxin-α+250 Is Associated with the Presence and Severity of Placental Inflammation among Preterm Births

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ABSTRACT

Histologic inflammation of placenta has been associated with increased risk for bronchopulmonary dysplasia and periventricular leukomalacia among preterm infants. Tumor necrosis factor-α (TNF-α) plays a central role in the regulation of inflammation. Some alleles of TNF (LT-α+250, TNF-α−308, and TNF-α−238) have been associated with susceptibility and/or severity of many diseases characterized by inflammation and/or involving the immune system. To determine whether alleles of TNF-α affect the risk and/or the severity of chorioamnionitis, we examined the placentas of 101 preterm births (birth weight ≤1250 g) for the presence of inflammation. Maternal and fetal chorioamnionitis (MCA and FCA, respectively) were graded for severity and staged for location of inflammatory infiltrate. Analysis for TNF-α alleles was done using PCR-restriction fragment length polymorphism technique on DNA extracted from infants' whole blood. MCA and FCA were seen in 45 and 38 placentas, respectively ($p = 0.64$). Genotypes of TNF-α−308 did not affect the development or the severity of placental inflammation. However, the AA genotype of LT-α+250 occurred more often when MCA and FCA were present compared with placentas without inflammation ($p = 0.016$ and $p = 0.007$, respectively). The GA genotype of TNF-α−238 was more common in placentas with severe MCA than with mild MCA ($p = 0.015$). The number of A alleles of LT-α+250 (GG = 0, GA = 1, AA = 2) correlated directly and significantly with grades and stages of MCA and FCA ($p < 0.05$). The AA genotype of LT-α+250 is associated with the development of chorioamnionitis among preterm births. The A allele of LT-α+250 seems to worsen the degree of placental inflammation. **(*Pediatr Res* 56: 94–98, 2004)**

Abbreviations
FCA, fetal chorioamnionitis
LT, lymphotoxin
MCA, maternal chorioamnionitis
PMN, polymorphonuclear leukocyte
TNF-α, tumor necrosis factor-α

Chorioamnionitis has long been recognized as an important cause of neonatal morbidity and mortality (1). It has been associated with preterm birth, preterm premature rupture of the fetal membranes, increased risk of neonatal sepsis, and death (2). Chorioamnionitis complicates up to 45% of all premature births with an incidence inversely proportional to gestational age (3,4). There is strong evidence to support a possible role for chorioamnionitis in neonatal encephalopathy and development of cerebral palsy among term infants (5–8). Preterm infants who are exposed to chorioamnionitis are at increased risk of periventricular leukomalacia, cerebral palsy (7,9), and chronic lung disease (10–13). Tumor necrosis factor-α (TNF-α) is an inducible cytokine and an important regulator of the inflammatory response with a broad range of proinflammatory and immunostimulatory actions (14,15). TNF-α is released by activated monocytes and/or macrophages early during an inflammatory reaction, and it induces the release of many other proinflammatory mediators such as IL-1, IL-6, and itself. Dysregulation of an inflammatory response resulting from an overproduction of proinflammatory cytokines may result in an exaggerated, uncontrolled inflammatory reaction; interfere with initiation of repair processes; and induce autoinjury with underlying tissue damage (16). Overproduction of TNF-α has been reported to occur in the presence of certain alleles within the regulatory region of the gene (17). Suscep-

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tibility and severity of many infectious diseases such as sepsis, cerebral malaria, leishmaniasis, and *Chlamydia* as well as autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and Crohn's disease are thought to have a genetic basis and have been linked to polymorphisms in the TNF-α gene regulatory region (18).

The TNF gene is located within the MHC on chromosome 6, along with two related genes encoding lymphotoxin-α (LT-α) and lymphotoxin-β (LT-β) (19). Lymphotoxin-α, also referred to as TNF-β, has actions similar to those of TNF-α, but its expression is more restricted.

A number of genetic polymorphisms in the TNF-α regulatory region resulting in the substitution of the nucleotide adenine (A) for guanine (G) at positions LT-α+250, TNF-α−238 and TNF-α−308 have been associated with higher levels of transcription of TNF-α gene (17,20,21). In this report, we examined whether the presence of the A allele of TNF-α−238, TNF-α−308, and LT-α+250 among preterm infants affected the risk of developing chorioamnionitis and the severity of placental inflammation.

METHODS

Infants were recruited from the neonatal intensive care nursery at Hutzel Hospital (Detroit, MI, U.S.A.). All infants who had a birth weight ≤1250 g were eligible for the study. After informed consent was obtained, 0.5 mL of whole blood was drawn either from an indwelling arterial or venous umbilical line or by venipuncture. DNA was extracted using the Genomic DNA purification Kit (Promega, Madison, WI, U.S.A.). Genotypic analysis for TNF-α polymorphic sites was performed using a PCR-restriction fragment length polymorphism technique, as previously described (22–24).

All placentas were processed in a standard manner. This included taking three full-thickness sections from the placental disc, three sections from the maternal surface, two sections of the cord, and a membrane roll. All sections were stained with hematoxylin and eosin. Maternal (MCA) and fetal (FCA) chorioamnionitis, when present, were graded on a scale of 0–2 on the basis of the severity and staged on a scale of 0–3 on the basis of the extent of the acute inflammatory infiltrate as manifested by polymorphonuclear leukocytes (PMNs).

MCA was graded as follows: grade 0, no inflammation; grade 1, not severe; and grade 2, severe inflammation defined as chorionic microabscesses in membranes and/or under the chorionic plate or a continuous band of confluent PMNs occupying more than one half of subchorionic fibrin or one revolution of the membrane roll. MCA staging included stage 0, no inflammation; stage 1, few PMNs in subchorionic plate fibrin and/or membranous chorionic trophoblast layer; stage 2, more than a few PMNs in the chorionic plate and/or chorionic connective tissue, amnion, or deciduus of membranes; and stage 3 (necrotizing chorioamnionitis) with karyorrhectic neutrophils, thickened amnionic eosinophilic basement membranes, and focal amnionic epithelial degeneration.

FCA was graded as follows: grade 0, no inflammation; grade 1, not severe; and grade 2, severe with many PMNs in chorionic surface vessels with attenuation and/or degeneration of vascular smooth muscle. FCA was staged as follows: stage 0, no inflammation; stage 1, PMNs in the wall of any chorionic surface vessel or the umbilical vein; stage 2, PMNs in one or both umbilical arteries; and stage 3 (necrotizing funisitis) with PMNs, cellular debris, mineralization, and/or neovascularization arranged in concentric bands, rings, or halo-like distribution around cord vessels. Two pathologists (S.M.J. and F.Q.) examined the placentas and graded and staged the inflammation without knowledge of the distribution of TNF-α alleles among infants.

Demographic variables and medical data were collected on all participants. Clinical chorioamnionitis was diagnosed in the presence of a temperature elevation of 37.8°C or higher and two or more of the following criteria: uterine tenderness, foul-smelling amniotic fluid, fetal tachycardia (fetal heart rate >160), and maternal leukocyte count >15,000 cells/mm^3 in the absence of another source of infection (25). The diagnosis of prolonged premature rupture of the fetal membranes was made when duration of membrane rupture was ≥18 h. The study was approved by the Institutional Review Board at Wayne State University School of Medicine.

Statistical analysis. χ^2 test was used to compare the genotypic frequencies and other categorical variables. Continuous variables were compared using *t* test. Kendall's tau_b correlation was used to examine the relationship between grades and stages of placental inflammation with genotypes of TNF-α. A $p \leq 0.05$ was set for all analyses.

RESULTS

A total of 101 preterm infants were recruited. MCA was detected in 45% of placentas and FCA in 38% of placentas ($p = 0.64$). There was no difference in maternal age, gravidity and parity, racial distribution, use of antenatal steroids, and infants' birth weight among infants with MCA and those without MCA ($p < 0.05$; Table 1). Mothers of infants with MCA were more likely to have a diagnosis of clinical chorioamnionitis, to have prolonged rupture of fetal membranes of ≥18 h, to have received antenatal antibiotics, and to deliver more immature infants than mothers of infants without MCA ($p < 0.05$; Table 1). The genotypic distribution of alleles of TNF-α−238 and TNF-α−308 was comparable among infants with and without MCA (Table 2). However, the AA genotype of LT-α+250 occurred significantly

Table 1. *Mother and infant characteristics*

	MCA present ($n = 45$)	No MCA ($n = 56$)
Maternal age (y)	27 ± 7	26 ± 7
Gravidity/parity	4 ± 3/2 ± 2	3 ± 2/2 ± 2
Black/white/Hispanic	13/32/0	10/42/4
Antenatal steroids	36 (80%)	44 (79%)
Clinical chorioamnionitis	15 (33%)*	3 (5%)*
PROM ≥18 h	17 (38%)*	4 (7%)*
Antibiotic therapy	34 (76%)*	16 (29%)*
Birth weight (g)	866 ± 201	930 ± 220
Gestational age (wk)	26 ± 4*	28 ± 2*

Values are mean (±SD) or number (percentage of patients). PROM, premature rupture of membranes.

* *T* test or χ^2 test, $p = 0.001$.

Table 2. *Genotypes of TNF and maternal placental inflammation**

	LT-α+250			TNF-α−238			TNF-α−308		
	AA	GA	GG	AA	GA	GG	AA	GA	GG
No MCA ($n = 56$)	25%	43%	32%	5%	4%	91%	5%	21%	74%
MCA ($n = 45$)	47%	42%	11%	–	11%	89%	2%	18%	80%
p value		0.016			0.107			0.626	
Grade 1 MCA ($n = 23$)	52%	35%	13%	–	–	100%	–	22%	78%
Grade 2 MCA ($n = 22$)	41%	50%	9%	–	23%	77%	4%	14%	82%
p value		0.583			0.015			0.477	
Frequency of A allele	A		G	A		G	A		G
No MCA ($n = 56$)	0.46		0.54	0.07		0.93	0.16		0.84
MCA ($n = 45$)	0.68		0.32	0.06		0.94	0.11		0.89
p value		0.004			0.866			0.418	

* χ^2 test.

Table 3. *Genotypes of TNF and fetal placental inflammation**

	LT-α+250			TNF-α−238			TNF-α−308		
	AA	GA	GG	AA	GA	GG	AA	GA	GG
No FCA ($n = 63$)	25%	43%	32%	5%	3%	92%	5%	22%	73%
FCA ($n = 38$)	50%	42%	8%	–	13%	87%	2%	16%	82%
p value		0.007			0.071			0.608	
Grade 1 FCA ($n = 36$)	50%	42%	8%	–	11%	89%	3%	14%	83%
Grade 2 FCA ($n = 2$)	–	50%	50%	–	50%	50%	–	50%	50%
p value		0.906			0.113			0.391	

* χ^2 test.

more often among infants with MCA compared with infants without MCA ($p = 0.016$; Table 2). Among infants with MCA, the GA genotype of TNF-α−238 occurred significantly more often among infants with severe MCA (grade 2) compared with infants with less severe MCA (grade 1; $p = 0.015$; Table 2). The frequency of the A allele of LT-α+250 was significantly higher among infants with MCA than infants without MCA ($p = 0.004$; Table 2). Similarly, the AA genotype of LT-α+250 was more likely to be present among infants with FCA than infants without FCA ($p = 0.007$; Table 3). Genotypes of TNF-α−238 and TNF-α−308 were comparable among infants with and without FCA ($p > 0.05$; Table 3). There was no difference in the genotypic distribution of all three alleles of TNF-α among infants with mild (grade 1) and severe (grade 2) FCA ($p > 0.05$; Table 3). There were significant positive correlations between the number of A alleles of LT-α+250 (GG = 0, GA = 1, and AA = 2) and grades of MCA as well as grades of FCA (Table 4). Similarly, there were significant direct correlations between the number of A alleles of LT-α+250 and stages of MCA and FCA (Table 4). The number of A alleles of TNF-α−238 did not correlate with grades of MCA ($p > 0.05$).

The genotypic distribution of alleles of LT-α+250, TNF-α−238 and TNF-α−308 was comparable among male ($n = 48$) and female ($n = 53$) infants ($p > 0.05$), as well as among infants of variable racial backgrounds (Table 5). The genotypic distribution of LT-α+250 among our infants was similar to that reported in adults (Table 6).

Table 4. *Severity of placental inflammation and LT-α+250 genotype**

	MCA		FCA	
	R	p value	R	p value
Grades of inflammation 0–2	0.236	0.013	0.274	0.006
Stages of inflammation 0–3	0.253	0.007	0.254	0.008

* Kendall's tau_b.

DISCUSSION

In this study, we examined the role of alleles in the regulatory region of TNF gene, specifically LT-α+250, TNF-α−238, and TNF-α−308, on the susceptibility and severity of placental inflammation among preterm births. We found that the presence of the AA genotype of LT-α+250 correlated significantly and directly with the susceptibility to develop MCA as well as FCA. Furthermore, the severity of placental inflammation correlated positively and significantly with the number of A alleles of LT-α+250. The GA genotype of TNF-α−238 occurred more often among infants with severe MCA (grade 2) compared with infants with less severe MCA (grade 1). However, we found no significant correlation between the number of A alleles of TNF-α−238 and placental inflammation.

The possible role of the A allele of LT-α+250 in human disease states has been reported by other investigators. Quasney *et al.* (24) found an increased frequency of the AA genotype of LT-α+250 among white children with Kawasaki disease compared with healthy control subjects. The AA genotype of LT-α+250 was associated with more severe psoriasis (26) and higher rate of mortality among children with bacteremia (27) and septic adults (22). Adult patients who had community-acquired pneumonia and were carriers of the AA LT-α+250 genotype were more likely to develop septic shock (28). Patients with multiple sclerosis were more likely to be carriers of the AA genotype of LT-α+250 compared with control subjects (29).

Table 5. *Racial distribution of TNF-α genotypes**

	LT-α+250			TNF-α−238			TNF-α−308		
	AA	GA	GG	AA	GA	GG	AA	GA	GG
White (n = 21)	48%	43%	9%	–	–	100%	5%	14%	81%
Black (n = 76)	29%	43%	28%	4%	10%	86%	4%	26%	70%
Hispanic (n = 4)	25%	75%	–	–	–	100%	–	–	100%

* χ^2 test, $p > 0.05$.

Table 6. *Genotypic distribution of LT-α+250 in premature infants compared with reported frequency in adults**

	AA	GA	GG
Premature infants in study	32%	45%	23%
Waterer *et al.* (28)	30%	48%	22%
Yende *et al.* (45)	39%	42%	19%

* χ^2 test, $p > 0.05$.

Reports on the possible role of TNF-α−308 and TNF-α−238 on susceptibility and severity of diseases involving the innate immune system have yielded conflicting results. The A allele of TNF-α−308 occurred more often among children who had Kawasaki disease and developed coronary artery abnormalities (24). Carriage of the A allele of TNF-α−308 was associated with worse outcome among patients with cerebral malaria (30), leishmaniasis (31), and severe sepsis (32) but had no effect on the susceptibility to or severity of rheumatoid arthritis (33), multiple sclerosis (34), or Crohn disease (35). Similarly, the A allele of TNF-α−238 was associated with severe silicosis (36) but was absent among patients with severe rheumatoid arthritis (33,37).

Although our data may suggest that carriage of the A allele of TNF-α−238 might be protective for histologic chorioamnionitis and may play a role in the severity of placental inflammation, the skewed distribution of genotypes of TNF-α−238 makes interpretation of this data difficult. Other investigators have suggested a possible protective role for the A allele of TNF-α−238 in disease conditions involving inflammation and/or the immune system (33,37–39). Perhaps a multicenter trial with a much larger sample size might shed more light on the functional significance of the A allele of TNF-α−238 in susceptibility and severity of chorioamnionitis.

We did not measure cord blood levels of TNF-α in our infants. Several other investigators had reported elevated levels of TNF-α in the presence of TNF-α−308, TNF-α−238, and LT-α+250 alleles (22,40,41). However, several intrapartum events, such as fetal distress, maternal fever, and/or intrapartum administration of antibiotics, may affect cord blood TNF-α levels (42–44).

So how could this association between the AA genotype of LT-α+250 and increased susceptibility to and severity of MCA and FCA be explained? Most diseases are the result of an interaction between an individual's genetic makeup, the environment, and the process that initiates injury. Disease is rarely regulated by the activity of a single gene. Thus, a more likely explanation for our observation is that the AA LT-α+250 genotype represents a marker for a cluster of co-regulated genes that are involved in the initiation and regulation of the inflammatory response. The TNF locus is located within the MHC, a dense cluster of highly polymorphic genes that determine an individual's immune response (45,46). The region encodes several proteins that are involved in inflammation, stress responses, and host defense. Furthermore, by affecting levels of transcription of the TNF-α gene, the A allele of LT-α+250 may precipitate a disequilibrium between the pro- and anti-inflammatory cytokines, promoting the development and progression of placental inflammation.

Because placental inflammation is commonly found among preterm births (45%), we entertained the possibility that the occurrence of the A allele/AA genotype of LT-α+250 may just be a marker of prematurity. However, the finding of a similar genotypic distribution of LT-α+250 among our preterm infants and that reported among adults (28,47) refuted that hypothesis.

To our knowledge, this is the first report to examine the role of genotypes of TNF on histologically documented placental inflammation among preterm infants. Simhan *et al.* (48) explored the relationship between carriage of the A allele of TNF-α−308 and clinical chorioamnionitis. They found a higher rate of clinical chorioamnionitis (24.4%) among pregnant women at term (37–42 wk gestation) who were carriers of the A allele of TNF-α−308 compared with noncarriers (7.4%). Clinical criteria for the diagnosis of chorioamnionitis are somewhat insensitive in detecting cases with histologic placental inflammation (49). Our findings were based on histologic diagnosis of placental inflammation. In our study, only 33% of patients with histologic evidence of chorioamnionitis had a diagnosis of clinical chorioamnionitis.

CONCLUSION

In summary, our data demonstrate that the AA genotype of LT-α+250 increases the susceptibility to chorioamnionitis among preterm births. The presence of the A allele of LT-α+250 worsens the severity of placental inflammation.

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