Association of two tumour necrosis factor gene polymorphisms with the incidence of severe intraventricular haemorrhage in preterm infants

A Heep, A C Schueller, E Kattner, M Kroll, J Sander, M Wisbauer, P Bartmann, F Stueber


The tumour necrosis factor (TNF) α and β gene pair is located in the human major histocompatibility complex between 6p21.1 and 6p21.3.1,2 Gene polymorphisms within the TNFα and TNFβ gene are well described.3,4 The extent of TNFα expression has been shown to be associated with the overall allele frequency and genotype distribution of the NcoI restriction fragment length polymorphism in the first intron of the TNFβ locus.5–7 In an in vitro endotoxin stimulation model, the extent of TNF expression has been correlated to the genotype of a single base polymorphism in the −308 promoter region of the TNFα gene.8,9 In contrast to this finding, the clinical importance of polymorphisms in the TNFα gene remains controversial. Survival in severe sepsis10 has not been associated with genotype distribution of single base mutation in the −308 promoter region of the TNFα gene.

Focussing on neonatal morbidity, neither the development of chronic lung disease of prematurity11 nor necrotising enterocolitis12 were correlated to genotype distribution of the TNFα gene, whereas the development of intraventricular haemorrhage (IVH) was correlated to the −308 promoter region polymorphism of the TNFα gene.13 Genetic determination of the individual inflammatory response may influence the susceptibility to poor outcome after systemic infection or severe tissue damage.14,15

Premature infants are at high risk of cerebral morbidity, which is associated with long term limitations and disability.16 Immaturity as well as a systemic perinatal inflammatory response17–19 play a major role in the pathogenesis of cerebral haemorrhage and white matter brain damage. Molecular markers predicting susceptibility to cerebral morbidity in premature infants are missing and thus prophylactic therapeutic strategies are still limited.

The aim of this study was to describe the frequency of two biallelic polymorphisms within the TNF locus in premature infants of <32 weeks gestation with severe IVH compared to healthy newborn infants without a history of cerebral morbidity.

METHODS

Study subjects

A retrospective cohort study was carried out on stored Guthrie blood spot cards stripped of all identifiers. The blood spot cards were from two groups of infants treated at the Department of Neonatology of the University of Bonn, Kinderkrankenhaus auf der Bult, Hannover, and Olgahospital Stuttgart, Stuttgart, Germany between January 1999 and December 2000.

Group A comprised 27 premature infants of <32 weeks gestational age with sonographic finding of severe IVH at postnatal day 7. The sonographic findings of IVH were classified using the criteria given by Volpe: (a) grade I (mild), germinal matrix haemorrhage with no or minimal IVH; (b) grade II (moderate), IVH (10–50% of ventricular area in parasagittal scan); (c) grade III (severe) (>50% of ventricular area in parasagittal scan); and (d) apparent periventricular haemorrhagic infarction. According to the classification given by Volpe, in our study severe IVH was defined as either grade III IVH or IVH with apparent periventricular haemorrhagic infarction.

Group B comprised 102 healthy newborn infants selected according to the study entry criteria (inborn patients, >32+0 weeks gestation, no signs of severe IVH on ultrasound examination at postnatal day 7).

All patients treated at the three study centres who met entry criteria were included into the study. TNF allele distribution of the study population was also compared to reference groups of healthy adult volunteers.20 Following the approval of the ethical committee of the University of Bonn, a three step study protocol was performed.

First, patients were recruited from the hospital patient charts according to study entry criteria. Second, stored Guthrie blood spot cards from the study population were
retrieved from the newborn screening laboratory. Third, the Guthrie blood spot cards were stripped of all identifiers and sent to the molecular genetic laboratory for analysis. Laboratory analysis and later data analysis were performed blind to personal data. All study procedures followed the 1975 Declaration of Helsinki revised in 2000.

**Laboratory investigation**

Human genomic DNA was extracted from stored Guthrie blood spot cards. For amplification of the 782 bp fragment of genomic DNA containing the polymorphic NcoI site within the TNFβ locus, blood spot cards were immersed in a mixture containing 0.334 μM each of primer 1 (5’ CCGTGTTCTGCTGCTTGGAGTTT 3’) and primer 2 (5’ AGAGGTTGGATGCTGTTGGGTTT 3’); PCR Premix E buffer (50 nM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, and 200 μM each of dATP, dTTP, dCTP, and dGTP) (Biorzym, Oldendorf, Germany); and ddH₂O. To amplify the 264 bp fragment of the TNFα promoter – 308 locus we used a mixture containing 0.4 μM each of primer 1 (5’ CCGTGCATCTGGTCTGGAAAGTTA 3’) and primer 2 (5’ CTGCACCTCCTGCTTGGTTT 3’); 200 μM each of dATP, dTTP, dCTP, and dGTP; and GeneAmp 10×buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂, and 0.01% w/v gelatine) (Perkin Elmer, Branchburg, NJ, USA). Filter papers with primers, buffer, and ddH₂O were exposed to the following two step incubation: four cycles of 3 min at 95 ºC and 3 min at 62 ºC. This program delivered DNA and reduced inhibition of PCR by free proteins and heavy metals. Afterwards, 1 U of Ampli Taq DNA polymerase (Perkin Elmer, Branchburg, NJ, USA) was added to the ice cold product was performed by analysing DNA melting curves in a gel electrophoresis showing the original 782 bp fragment for male patients of group A compared to male patients of group B (p<0.04).

Table 3 shows the TNFβ1/2 and TNFα promoter – 308 allele distribution of group A compared to group B. The overall incidence of the TNFβ allele was significantly higher in group A compared to group B (p<0.01). In male patients, TNFβ allele frequency was significantly elevated in group A compared to group B (table 4, p<0.01), whereas no difference was found when comparing the TNFβ1/2 allele frequency of female patients (group A v group B, p<0.28). No difference was found for the TNFα promoter – 308 allele frequency within the groups.

The Hardy-Weinberg equilibrium of genotype distribution was estimated to compare group B and the reference groups. The genotype distribution of group B did not differ from the reference groups (TNFβ sc: p<0.66; TNFα – 308: p<0.94).

**DISCUSSION**

TNFα plays a pivotal role in the proinflammatory cytokine cascade.

The biallelic NcoI polymorphism within the TNF locus was shown to be a genomic marker for increased TNFα expression and outcome in adult patients with severe sepsis.

In the model of lipopolysaccharide (LPS) induced TNFα production by human monocytes in an ex vivo culture enhanced TNFα expression was found in relation to TNFα genotype.6

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**Table 1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group A (n, %)</th>
<th>Group B (n, %)</th>
<th>Reference (n, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFβ1</td>
<td>1/1</td>
<td>1 (3,7%)</td>
<td>18 (17,7%)</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>8 (29,6%)</td>
<td>39 (38,2%)</td>
</tr>
<tr>
<td></td>
<td>2/2</td>
<td>18 (66,7%)</td>
<td>45 (44,1%)</td>
</tr>
<tr>
<td>Total</td>
<td>27 (100%)</td>
<td>102 (100%)</td>
<td>252 (100%)</td>
</tr>
<tr>
<td>p Value</td>
<td>p&lt;0.06</td>
<td>p&lt;0.66</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>1/1</td>
<td>19 (70,4%)</td>
<td>68 (66,7%)</td>
</tr>
<tr>
<td>promoter</td>
<td>308</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2</td>
<td>7 (25,9%)</td>
<td>29 (28,4%)</td>
<td>69 (29,6%)</td>
</tr>
<tr>
<td>2/2</td>
<td>58 (19,6%)</td>
<td>213 (21,5%)</td>
<td>219 (21,9%)</td>
</tr>
<tr>
<td>Total</td>
<td>27 (100%)</td>
<td>102 (100%)</td>
<td>233 (100%)</td>
</tr>
<tr>
<td>p Value</td>
<td>p&lt;0.92</td>
<td>p&lt;0.94</td>
<td></td>
</tr>
</tbody>
</table>

Reference groups: *group 1 TNFβ gene (n = 252); †group 2 TNFα – 308 gene (n = 233)
Studies on TNF gene polymorphism in neurodegenerative diseases demonstrate that polymorphism in the TNFα gene (C850T) is a risk factor for the development of Alzheimer’s disease and vascular dementia. In paediatric patients with cerebral malaria, the TNFα gene (C850T) polymorphism was found to be a prognostic marker for disease progression. However, focusing on polymorphism in the TNFα gene and specific neonatal morbidity only limited reports are available.

The ability of the neonate to react on LPS exposure with enhanced TNFα production was demonstrated by experimental data on TNFα release following LPS stimulation in an ex vivo cord blood culture model. Studies on evaluation of TNFα for the early diagnosis of neonatal infection indicate that high expression of TNFα measured in the serum appears to be a highly sensitive and specific marker of sepsis in the early postnatal period. However, focusing on polymorphism in the TNFα gene and specific neonatal morbidity only limited reports are available.

In a study on neonates with proven sepsis, the biallelic NcoI polymorphism within the TNFα locus was not found to be a prognostic marker for disease progression.

Induction of TNFα release in the brain following hypoxic ischaemic encephalopathy or IVH was shown by TNFα measurements in the CSF and CSF/plasma ratios in newborn infants. From this finding, one may assume that local enhanced inflammatory reaction is of certain importance in the pathogenesis of cerebral morbidity in the newborn infant.

The incidence of IVH in premature infants is independently related to gestational age and different perinatal morbidity. There is evidence that inflammatory umbilical cord lesions and elevated proinflammatory cytokine levels in the amniotic fluid or in the cord blood are associated with cerebral morbidity in newborn infants. Intrauterine T cell activation and increased inflammatory activity has been shown to be related to early postnatal changes of the cerebral white matter on magnetic resonance imaging. In a clinical study on preterm infants of <28 weeks gestation, early systemic inflammatory neonatal response measured by serum IL-6 was significantly correlated with the development of severe IVH independent of gestational age.

Studies on human cerebral microvascular endothelial cells indicate that these are capable of up-regulating inflammatory endothelial mediators in response to proinflammatory cytokines or ischaemia. Consecutive pathophysiological steps are vasoparalysis and activation of microglial cytokine expression. Haemodynamic disturbances are known to be related to cerebral morbidity in preterm infants. Recently, a study showed correlation of systemic fetal inflammation (chorioamnionitis, elevated proinflammatory cord blood cytokines) with reduction of systemic blood pressure, cardiac output, and increased incidence of severe IVH in premature infants.

Taking the experimental and clinical findings as a basis, one may speculate that polymorphisms in the TNF genome that distinctly contribute to TNFα release may lead to haemodynamic changes and by inducing cytotoxic cerebral microvascular damage thereby lead to severe IVH.

We studied two different gene polymorphisms within the TNFα and TNFβ gene to investigate genotype distribution in correlation to the incidence of severe IVH in preterm infants. The study group was stratified by gender as male gender is a known perinatal risk factor in the pathogenesis of severe IVH.

One previous report describes correlation of single base polymorphism in the –308 promoter region of the TNFα gene with the incidence of IVH in premature infants. In contrast to this finding, in our study, the distribution of TNFα promoter –308 polymorphism was not different within the study groups and no difference was found with regards to gender.

The overall frequency of the TNFβ2 allele in group A was found to be elevated compared to group B. The TNFβ2 genotype in male patients indicated a significant risk of severe IVH in patients of <32 weeks gestation. Although the study results are limited by the small number of patients included, it extends observations which associate the TNFβ2 allele distribution with illness severity of patients in intensive care. Our study is the first to indicate that a predominance of the TNFβ2 allele and genotype of a proinflammatory cytokine such as TNF in male gender is linked to a major neuropathological outcome parameter in premature infants.

ETIOLOGIC research, stratifying premature infants at risk of cerebral morbidity following perinatal risk factors, is necessary to elucidate the significance of the genetic background as an independently predictive variable in the complex pathophysiological sequence leading to cerebral morbidity in preterm infants.

ACKNOWLEDGEMENTS
We gratefully acknowledge the skilled technical assistance of Alexandra Casalder and the revision of genetic statistics by Rolf Fimmers.
Table 4  TNFβ1/2 and TNFα promoter –308 allele distribution of male and female patients (group A vs group B)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Group A male, n = 30 (n, %)</th>
<th>Group B male, n = 114 (n, %)</th>
<th>Group A female, n = 24 (n, %)</th>
<th>Group B female, n = 90 (n, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFβ1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4 (13, 3%)</td>
<td>42 (36, 8%)</td>
<td>6 (25%)</td>
<td>33 (36, 7%)</td>
</tr>
<tr>
<td>2</td>
<td>26 (86, 7%)</td>
<td>72 (63, 2%)</td>
<td>18 (75%)</td>
<td>57 (63, 3%)</td>
</tr>
<tr>
<td>p Value</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα promoter –308</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>26 (86, 7%)</td>
<td>92 (80, 7%)</td>
<td>19 (79, 2%)</td>
<td>73 (81, 1%)</td>
</tr>
<tr>
<td>2</td>
<td>4 (13, 3%)</td>
<td>22 (19, 3%)</td>
<td>5 (20, 8%)</td>
<td>17 (18, 9%)</td>
</tr>
<tr>
<td>p Value</td>
<td>0.45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

REFERENCES


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doi: 10.1136/jmg.2004.021378