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

LETTER TO JMG

The tumour necrosis factor (TNF) α and β gene pair is located in the human major histocompatibility complex between 6p21.1 and 6p21.3. Genes polymorphisms within the TNFα and TNFβ gene are well described. The extent of TNFα expression has been shown to be associated with the overall allele frequency and genotype distribution of the Ncol restriction fragment length polymorphism in the first intron of the TNFβ locus. 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. In contrast to this finding, the clinical importance of polymorphisms of the TNFα gene remains controversial. Survival in severe sepsis has not been associated with genotype distribution of single base mutation in the −308 promoter region of the TNFα gene.

To clarify on neonatal morbidity, neither the development of chronic lung disease of prematurity nor necrotising enterocolitis 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. Genetic determination of the individual inflammatory response may influence the susceptibility to poor outcome after systemic infection or severe tissue damage. Premature infants are at high risk of cerebral morbidity, which is associated with long term limitations and disability. Immaturity as well as a systemic perinatal inflammatory response 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. The study was performed in three steps. Group A comprised 27 premature infants of <32 weeks gestational age with sonographic findings 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. 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

Key points

- There are no molecular markers predicting susceptibility to cerebral morbidity in premature infants and therapeutic strategies are still limited. Genetic polymorphisms in the tumour necrosis factor (TNF) gene modify TNF expression. A systemic perinatal inflammatory response is known to be a risk factor for severe intraventricular haemorrhage (IVH) in preterm infants.
- We studied the frequency of biallelic polymorphisms of the TNFα promoter region and the Ncol polymorphism of the TNFβ gene in premature infants with severe IVH. The overall allele frequency and genotype distribution of the −308 TNFα polymorphism were comparable with values found in controls.
- The overall incidence of the TNFβ2 allele was higher in the IVH group compared to the control group. Genotype distribution of a polymorphic site within the TNFβ locus in the male patient group significantly differed from distribution in the control group. Male patients showed a significantly higher prevalence of the homozygous genotype for the TNFβ2 allele.
- Our study results provide the first molecular link between TNFβ gene polymorphism and the incidence of severe IVH in preterm infants.

Abbreviations: IVH, intraventricular haemorrhage; LPS, lipopolysaccharide; TNF, tumour necrosis factor


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 according 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 hemorraghic infarction.

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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’ CGTGCTTCTGCTGCTGGTACTGA 3’) and primer 2 (5’ AGAGGGGGTTGACTGGTTGCTGC 3’); PCR Premix E buffer (50 mM Tris-HCL, 50 mM KCl, 25 mM MgCl₂, and 200 μM each of dATP, dTTP, dCTP, and dGTP) (Bionzym, 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’ CCTGCATCTCCTTGGAAAGTTA 3’) and primer 2 (5’ CTGACACCTCCTGCTCGGTTTT 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°C and 3 min at 62°C. This program delivered DNA and reduced bromide. NcoI digestion was then performed with 5 mM NcoI (TIB MOLBIOL, Berlin, Germany). Therefore, we used 0.15 ml of amplified products by gel electrophoresis on a 1.6% agarose gel stained with 0.7 μg/ml ethidium bromide. NcoI digestion was then performed with 5 μl amplified DNA product. Digested DNA was again analysed by gel electrophoresis showing the original 782 bp fragment (homozygous patients for allele TNFβ2, lacking the NcoI site), three fragments of 782, 586, and 196 bp (heterozygous patients), or two fragments of 586 and 196 bp (patients homozygous for the allele TNFβ1). The PCR protocol for the TNFα product included denaturation followed by 43 cycles of 20 s denaturation at 95°C, 60 s annealing at 64°C, and 30 s extension at 72°C. Final extension was performed for 7 min at 72°C. Gel electrophoresis showed the 264 bp fragment. Genotyping of the TNFα product was performed by analysing DNA melting curves in a real time PCR system (LightCycler, Roche, Mannheim, Germany). Therefore, we used 0.15 μM each of anchor (5’ RED640-CAAAAACCTTATGGCTCATTCTATTTTGGGACPh 3’) and sensor (5’ AACCCCCGTCTCCATGGCCC-x 3’) oligonucleotides (TIB MOLBIOL, Berlin, Germany). The latter included at position 10 the variable –308 nucleotide of the promoter region TNFα and was fully complementary only to the PCR product of TNF allele 1. Denaturation of the probes by rapid heating up to 95°C (ΔC°s = 20) was directly followed by a 25 s annealing step at 45°C while the fluorophore coupled oligonucleotides hybridised to adjacent regions of the target DNA. Fluorescin of the sensors 3’end which was excited by an external light source was now able to activate LightCycler RED640 on the 5’ end of the anchor. The excited RED640 then emitted measurable light at 640 nm. During slow denaturation (95°C with ΔC°s = 0.2) binding of anchor and sensor to target DNA melts and fluorescence could not be observed. Since the sensor bound much more tightly to the PCR product of the TNFα allele 1, the melting temperature was higher and could easily be distinguished from that of the TNFα allele 2. In case of heterozygosity two melting curve peaks were detectable.

**Statistical analysis**

Statistical analysis of the genotype distribution and allele frequency was carried out by χ² test with two sided p values to compare values between groups of patients. The groups were compared by estimating Hardy-Weinberg equilibrium.

**RESULTS**

Table 1 shows the genotype distribution of the TNFβ and TNFα – 308 genes in group A compared to group B and reference groups. Regarding the NcoI polymorphism of the TNFβ gene, the genotype distribution in group A was no different from that of group B (p<0.06). No difference between the groups was found for TNFα – 308 genotype distribution.

When stratified by gender (table 2) a significant difference for the TNFβ genotype distribution was found 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, TNFβ2 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: 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.4

**Table 1** Genotype distribution of TNFβ and TNFα – 308 variants in group A (n = 27) compared to group B (n = 102) and reference groups

<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>TNFβ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1</td>
<td>1 (3, 7%)</td>
<td>18 (17, 7%)</td>
<td>35 (13, 9%)</td>
</tr>
<tr>
<td>1/2</td>
<td>8 (29, 6%)</td>
<td>39 (38, 2%)</td>
<td>98 (38, 9%)</td>
</tr>
<tr>
<td>2/2</td>
<td>18 (66, 7%)</td>
<td>45 (44, 1%)</td>
<td>119 (47, 2%)</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>&lt;0.06</td>
<td>&lt;0.66</td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>promoter</td>
<td>308</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1</td>
<td>19 (70, 4%)</td>
<td>68 (66, 7%)</td>
<td>151 (64, 8%)</td>
</tr>
<tr>
<td>2/2</td>
<td>7 (25, 9%)</td>
<td>29 (28, 4%)</td>
<td>69 (29, 6%)</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>&lt;0.92</td>
<td>&lt;0.94</td>
<td></td>
</tr>
</tbody>
</table>

Reference groups: a) group 1 TNFβ gene (n = 252); b) group 2 TNFα – 308 gene (n = 235)
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 cerebral malaria was associated with a predominance of the homozygous TNF2 genotype.

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 and TNFβ gene and specific neonatal morbidity only limited reports are available.

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 Casalter 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 v 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</td>
<td>1</td>
<td>4 (13, 3%)</td>
<td>42 (36, 8%)</td>
<td>6 (25%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26 (86, 7%)</td>
<td>72 (63, 2%)</td>
<td>18 (75%)</td>
</tr>
<tr>
<td>p Value</td>
<td>0.01</td>
<td></td>
<td></td>
<td>0.28</td>
</tr>
<tr>
<td>TNFα promoter –308</td>
<td>1</td>
<td>26 (86, 7%)</td>
<td>92 (80, 7%)</td>
<td>19 (79, 2%)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4 (13, 3%)</td>
<td>22 (19, 3%)</td>
<td>5 (20, 8%)</td>
</tr>
<tr>
<td>p Value</td>
<td>0.45</td>
<td></td>
<td></td>
<td>0.83</td>
</tr>
</tbody>
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Competing interests: none declared

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