Plasma Tumor Necrosis Factor-alpha (TNF-α) Levels Correlate with Disease Severity in Spastic Diplegia, Triplegia, and Quadriplegia in Children with Cerebral Palsy

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Inflammatory responses *in utero* and in neonates have been involved in the development of white matter lesions. This study aimed to investigate the role of tumor necrosis factor-alpha (TNF-α) in spastic cerebral palsy.

Material/Methods: Plasma TNF-α was measured by ELISA in 54 children with spastic cerebral palsy and 28 aged-matched controls. Both groups were split into age subgroups (1–3 *vs.* 4–12). Gross motor function and activities of daily living were assessed on enrollment and after 6 months of rehabilitation.

Results: TNF-α was higher in patients with cerebral palsy than in controls in young (P<0.001) and older subjects (P<0.001). TNF-α levels were comparable in both control subgroups (P=0.819). Younger patients with cerebral palsy had significantly higher TNF-α levels compared with older ones (P<0.001). Pre-rehabilitation TNF-α levels correlated with improvements in activities of daily living after rehabilitation (P<0.001).

Conclusions: Children with cerebral palsy showed higher plasma levels of TNF-α than controls. In addition, pre-treatment TNF-α levels were correlated with the improvements after rehabilitation therapy.

MeSH Keywords: **Aphonia • Cerebral Palsy • Tumor Necrosis Factor-alpha**

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Background

Cerebral palsy is a collective term for motor deficits resulting from non-progressive abnormalities in the immature brain occurring in about 2 of every 1000 live births in developed countries [1]. Inflammatory responses *in utero* and in neonates have been involved in the development of the white matter lesions that cause cerebral palsy [2–6]. Infection *in utero* can cause damaging perturbations in inflammatory responses, and infants who develop an immune response *in utero* are at higher risk of cerebral lesions [5]. Interleukin (IL)-6 and IL-1β levels in umbilical cord plasma, amniotic fluid, and cerebrospinal fluid are elevated in neonates with periventricular leukomalacia-associated lesions [7,8], but this is controversial [9].

Inflammation at or shortly after birth is associated with subnormal development in very preterm infants [10]. Perinatal cytokine levels were often reported to predict the risk of cerebral palsy in preterm children [11–13]. In addition, polymorphisms in TNF-α and IL-1β have been found to represent a genetic susceptibility to white matter damage and consequently cerebral palsy [2,14,15]. However, a meta-analysis demonstrated that cerebral palsy is associated with a genetic polymorphism in IL-6, but not with TNF-α or any other cytokine [16,17]. Although the precise immune responses involved in the development of cerebral palsy remain to be fully characterized, aberrant inflammatory responses may continue to influence the reconstruction of cerebral function after birth [18–20].

TNF-α has widespread biological functions, activating neutrophils to eliminate pathogenic microorganisms, stimulating mononuclear cells to produce IL-1β and IL-6, and activating B lymphocytes to produce antibodies. TNF-α is secreted by astrocytes, microglia, blood-borne macrophages, and vascular endothelial cells in the central nervous system [21]. TNF-α can increase vasopermeability by promoting endothelial cell proliferation, activation, and apoptosis [22,23]. TNF-α has been reported to cause increased blood-brain barrier permeability, participating in the development of cerebral edema [24,25]. Activated microglia can induce blood-brain barrier dysfunction by releasing TNF-α [21,26].

Although there is evidence suggesting that high levels of TNF-α are associated with cerebral palsy [9–11,14,15,20], little is known about age-related and function-related changes in TNF-α. In the present study, levels of plasma TNF-α in children with cerebral palsy were measured to assess the hypothesis that TNF-α levels can act as a marker of both cerebral palsy presence and cerebral palsy severity. In addition, patients were evaluated after rehabilitation therapy, and the pre-therapy levels of TNF-α were compared with their response to therapy.

Material and Methods

Subjects

Children with clinically confirmed spastic cerebral palsy were recruited from the Department of Rehabilitation, the Second Affiliated Hospital of Anhui Medical University and the Provincial Children's Hospital between December 2012 and August 2013. Cerebral palsy was diagnosed according to the definition, classification, and diagnostic criteria recommended by the Second National Children Rehabilitation Conference & the Ninth Chinese Cerebral Palsy Children Rehabilitation Academic Conference in Chang sha, China in 2009. The inclusion criteria were: 1) aged 1–12 years; and 2) stable health. Exclusion criteria were: 1) history of epilepsy or fever in the preceding two months; 2) inflammatory response syndrome; 3) diseases that could elevate TNF-α levels for other reasons, such as progressive amyotrophy, encephalomyelitis, severe malnutrition, myasthenia gravis, epilepsy, vision/hearing disorders, or other severe pediatric diseases; or 4) other endocrine disorders, metabolic disorders, autoimmune diseases, or genetic diseases.

Study design

The enrolled subjects with cerebral palsy were divided into 2 subgroups: those aged 1 to 3 years (n=27) and those aged 4 to 12 years (n=27). Twenty-eight control subjects free of brain disorders were recruited according to the same inclusion/exclusion criteria and were individually matched based on sex and age to the subjects with cerebral palsy. The age cut-off point was selected because the blood-brain barrier mature around 3 years of age, preventing plasma inflammatory factors from damaging the brain [27].

Blood samples were provided by all participants and the plasma levels of TNF-α were measured. Patient gross motor function and activities of daily living were evaluated before and after six months of individualized physical and speech therapy. Control participant gross motor function and activities of daily living was evaluated over the same period.

The study was approved by the Ethics Committee of Anhui Medical University (K201209), and informed consents were obtained from the children's legal guardians.

Clinical assessment

Participant gross motor function was evaluated by the gross motor function classification system (GMFCS) [28], which is a 5-level clinical classification system describing the gross motor function on the basis of self-initiated movement abilities. Activities of daily living were assessed by the Lawton IADL

scale [8]. All assessments were administered by the chief physician of the department (30 years of clinical experience in diagnosing, evaluating, and treating cerebral palsy).

GMFCS motor deficit severity was classified into levels I (for the highest level of activity) to V (for the lowest level of activity). The activities of daily living was assessed at the beginning of the study and again after six months of individualized physical and speech therapy, and the difference in score was recorded.

TNF-α analysis from venous blood

Venous blood samples (3 ml) were collected in pyrogen-free tubes. Plasma was separated at 6,000 g for 5 min, and stored at –30°C. Plasma TNF-α levels were measured by ELISA (BMS223/4, Bender MedSystems GmbH, Vienna, Austria).

Statistical analysis

All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Continuous data are expressed as mean ± standard deviation (SD). Categorical data were analyzed using the chi-square test. Continuous data were analyzed by multivariate analysis by non-conditional logistic regression analysis between all subjects with cerebral palsy and controls. TNF-α levels in each group were compared using the Student's t test. Correlation analysis between TNF-α levels and motor functions was performed using the Spearman correlation analysis. The correlation between TNF-α levels and improvement of activities of daily living was assessed by Pearson correlation analysis. A P-value <0.05 was considered statistically significant.

Results

Characteristics of the subjects

There was no significant difference in height, body weight, age, and sex between subjects with cerebral palsy and healthy controls (Tables 1, 2).

Plasma levels of TNF-α

The mean plasma TNF-α levels of subjects with cerebral palsy (21.4±6.2 pg/ml) was significantly higher than in controls (11.2±4.1 pg/ml) (P<0.001). This difference was present in both age subgroups (younger: 25.0±4.9 pg/ml *vs.* 11.4±3.4 pg/ml, P<0.001; and older: 17.8±5.2 pg/ml *vs.* 11.0±4.9 pg/ml, P<0.001). While the levels of TNF-α in the plasma of both control subgroups were comparable (P=0.819), younger subjects with cerebral palsy had significantly higher plasma TNF-α levels than older subjects with cerebral palsy (P<0.001) (Table 2).

Multivariate analysis revealed that although neither age nor sex were significantly correlated with cerebral palsy, plasma levels of TNF-α were significantly correlated with cerebral palsy (OR=1.605, 95%CI 1.302–1.979, P<0.001) (Table 3).

Correlation between TNF-α levels and cerebral palsy severity

There was no correlation between plasma TNF-α levels and GMFCS in the entire group of subjects with cerebral palsy. However, plasma TNF-α levels correlated significantly with GMFCS in the subgroups of patients with spastic triplegia or quadriplegia cerebral palsy (r=0.525, P=0.038), and spastic diplegia cerebral palsy (r=0.521, P=0.001) (Table 4). Nevertheless, a general trend toward an association in the whole group could be observed.

Pre-rehabilitation plasma TNF-α levels were also significantly correlated with the improvements in activities of daily living after 6 months of comprehensive rehabilitation in all subjects with cerebral palsy (r=–0.593, P<0.001) (Table 4). Similar results were observed in subjects with spastic triplegia or quadriplegia cerebral palsy (r=–0.839, P<0.001), and spastic diplegia cerebral palsy (r=–0.796, P<0.001).

Discussion

As early as 2007, Dammann et al. proposed that persistent activation or accumulation of inflammatory cytokines may impede the reconstruction of brain function following brain injury, therefore promoting cerebral palsy [29]. These pathological changes disturb the maturation of oligodendrocytes, limit neuronal regeneration, and impair synapse formation [30,31].

In this study, we measured the plasma levels of TNF-α in 54 children with spastic cerebral palsy, and in 28 age-matched healthy controls, and examined whether the levels of TNF-α were associated with the presence of cerebral palsy and the degree of functional disturbance in cerebral palsy. The plasma levels of TNF-α were significantly higher in children with spastic cerebral palsy, and significantly higher in children with cerebral palsy aged 1–3 than children aged 4–12. These results suggest that immunological abnormalities, while more pronounced before the age of four, may persist in cerebral palsy throughout childhood.

Enormous production of inflammatory cytokines including TNF-α and IL-1β has been previously detected in vulnerable brain regions of premature infants with white matter injury [32]. High levels of IL-1β, IL-6, IL-8, IL-9, and TNF-α have also been detected in the perinatal peripheral blood of full-term [11] and preterm [18] subjects with cerebral palsy, but this is controversial [12].

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Table 1. Demographic and clinical characteristics of subjects with cerebral palsy and controls.

Characteristics	CP (n=54)	Controls (n=28)	P
Sex			0.621
Male	32 (59.3%)	15 (53.6%)	
Female	22 (40.7%)	13 (46.4%)	
Age (year)	3.7±2.3	4.6±3.1	0.190
Height (cm)	97.8±16.9	105.3±20.0	0.075
Weight (kg)	16.2±5.6	19.2±8.2	0.051
BMI	16.6±1.6	16.7±1.2	0.892
TNF-a (pg/ml)	21.4±6.2	11.2±4.1	<0.001
CP type			
Spastic triplegia or quadriplegia	32 (59.3%)		
Spastic diplegia	16 (29.6%)		
Spastic monoplegia	5 (9.2%)		
Spastic hemiplegia	1 (1.9%)		
GMFCS			
I	5 (9.3%)		
II	16 (29.6)%		
III	14 (25.9%)		
IV	9 (16.7%)		
V	10 (18.5%)		

CP – cerebral palsy; BMI – body mass index; TNF-α – tumor necrosis factor a; GMFCS – gross motor function classification system.

Table 2. Demographic and clinical characteristics of subjects, by age group.

| Characteristics | Younger children (1 to 3 years) | | | Older children (4 to 12 years) | | |
	CP (%) (n=27)	Controls (%) (n=14)	P	CP (%) (n=27)	Controls (%) (n=14)	P
Sex						
Male	17 (63.0%)	7 (50.0%)	0.424	15 (65.6%)	8 (57.1%)	0.923
Female	10 (37.0%)	7 (50.0%)		12 (44.4%)	6 (42.9%)	
Age (year)	1.9±0.6	2.2±0.6	0.093	5.5±1.9	6.9±2.7	0.058
Height (cm)	84.7±10.2	90.7±9.0	0.700	110.6±11.9	119.8±17.1	0.056
Weight (kg)	12.6±2.8	14.1±2.9	0.112	19.7±5.5	24.3±8.6	0.086
BMI	17.4± 1.7	16.9± 0.8	0.273	15.9± 1.0	16.4± 1.5	0.197
TNF-α (pg/ml)	25.0±4.9	11.4±3.3	<0.001	17.8±5.2	11.0±4.9	0.001

CP – cerebral palsy; BMI – body mass index; TNF-α – tumor necrosis factor α; GMFCS – gross motor function classification system.

In the present study, only children with no history of fever, epilepsy, or inflammatory disorders in the previous two months were enrolled. Children under the age of four with spastic cerebral palsy had higher levels of plasma TNF-α compared with older children. This observation suggests that the inflammatory response may decrease with age, while the symptoms of cerebral palsy do not necessarily improve [1]. Additionally, higher baseline levels of TNF-α might be a marker of more extensive brain damage and of a weaker than average response to this form of therapy. Since therapies targeting activated microglia and astrocytes or anti-cytokine treatments may help reduce the injury of cerebral palsy [33], therapeutic interventions

Table 3. Multivariate analysis of TNF-α between all cerebral palsy and controls.

	P	OR	95% CI	
			Lower	Upper
TNF-α	<0.001	1.605	1.302	1.979
Age	0.132	1.244	0.937	1.651
Sex	0.901	0.913	0.219	3.813

OR – odds ratio; CI – confidence interval; TNF-α – tumor necrosis factor α. Sex was analyzed as male *vs*. female.

Table 4. Relevance analysis between TNF-α levels and motor functions in spastic cerebral palsy.

Parameters	n	r	P
GMFS			
All CP	54	0.269	0.051
Spastic triplegia or quadriplegia	32 (59.3%)	0.525	0.038
Spastic diplegia	16 (29.6%)	0.521	0.001
Spastic monoplegia	5 (9.2%)	–	–
Spastic hemiplegia	1 (1.9%)	–	–
Improvement of ADL			
All subjects	54	−0.593	<0.001
Spastic triplegia or quadriplegia	32 (59.3%)	−0.839	<0.001
Spastic diplegia	16 (29.6%)	−0.796	<0.001
Spastic monoplegia	5 (9.2%)	–	–
Spastic hemiplegia	1 (1.9%)	–	–

'–' – number of cases in these categories was too small to carry out analysis. CP – cerebral palsy; ADL – activities of daily living; GMFCS – gross motor function classification system.

designed to reduce inflammation may yield beneficial results in children throughout childhood. Recently, Alcaraz et al. [34] reported the use of platelet-rich plasma for the treatment of a patient with cerebral palsy, leading to improvements in the cognitive score and motor functions. Another study reported decreased pro-inflammatory factors after allogeneic umbilical cord blood transplantation [35]. However, the exact mechanisms of these approaches require further study.

To investigate the relationship between TNF-α and cerebral palsy severity, the gross motor function was evaluated in all subjects. Although TNF-α levels were not associated with GMFCS in the entire sample, TNF-α levels were significantly correlated with GMFCS in patients with spastic triplegia and quadriplegia, and with diplegia, supporting the hypothesis that circulating levels of TNF-α can reflect the severity of motor dysfunction in some types of spastic cerebral palsy.

Inflammation and intrauterine infection have been reported to activate microglia of the central nervous system, changing the function of neurons and glial cells, and causing cytotoxicity, edema, and brain injury in the fetal and neonatal brain [36]. Elevated levels of circulating TNF-α found in the present study suggest that this inflammatory process persists throughout childhood. A variety of different forms of brain injury have been associated with cerebral palsy including cerebellar atrophy, diffuse cortical atrophy, periventricular leukomalacia , thin corpus callosum, and border-zone infarction [37,38]. The present study suggests that the severity of illness was correlated to TNF-α levels in spastic triplegia and quadriplegia, and in diplegia.

The present study is not without limitations. Indeed, the sample size was small, preventing us from performing subgroup analyses. In addition, the follow-up was short. To confirm the capacity of TNF-α to represent a marker of disease severity, or

predict outcome of interventions, long-term follow up of a larger sample will be necessary. In addition, no comprehensive panel of inflammatory biomarkers was assessed. Nevertheless, a correlation between TNF-α levels and response to rehabilitation was observed. If this correlation is confirmed, then the hypothesis that pharmaceutical therapies to reduce inflammation in infants diagnosed with cerebral palsy may lessen the symptoms of cerebral palsy may deserve further attention. Nevertheless, previous attempts to intervene with the development of cerebral palsy have not yielded long term benefits [39], but a better understanding of the aberrant immune response will undoubtedly help to develop potential therapeutic strategies for early treatment of cerebral palsy. Finally, the neuroplasticity phenomenon might be involved in the increase if TNF-α levels, but further study is necessary to address this issue.

Conclusions

In conclusion, children with cerebral palsy showed higher plasma levels of TNF-α than controls. In addition, pre-treatment TNF-α levels were correlated with the improvements after rehabilitation therapy.

Acknowledgments

Conflicting Interests

The authors declare that they have no conflict of interest.

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