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Nerve Growth Factor (NGF) up-regulation in the cerebrospinal fluid of newborns with myelomeningocele

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Abstract. – BACKGROUND: Neurotrophic factors, such as Nerve Growth Factor (NGF), play a key role in the stimulation of sprouting, synaptic plasticity, and reorganization after spinal cord damage.

AIM: The aim of this study was to investigate the expression of nerve growth factor (NGF) in the cerebrospinal fluid (CSF) of newborns with myelomeningocele (MMC) and to determine its correlation with this spinal malformation.

PATIENTS AND METHODS: To measure the expression of NGF, we collected CSF samples of 14 newborns with MMC taken immediately before the neurosurgical correction of the spinal malformation and of 14 matched controls. Endogenous NGF levels were quantified using a two-site immuno-enzymatic assay. The statistical analysis was performed using the Mann-Whitney two-tailed two-sample test.

RESULTS: In the CSF of patients with MMC, NGF levels showed a significant increase compared to the mean levels of the control group (63.05 ± 7.3 vs 18.32 ± 4.5 pg/mL; ($p < 0.001$). No correlation was found between NGF expression and different types of MMC malformation, such as the level of spinal lesion and the association with Chiari II syndrome.

CONCLUSIONS: Our study shows an over-expression of NGF in the CSF of newborns with MMC. The observed pattern of NGF up-regulation in this subset of patients may stimulate axonal sprouting and synaptic reorganization of the damaged neural cells at the site of spinal cord injury, thereby representing an important biochemical marker of spinal cord damage in MMC patients.

Key Words:
Myelomeningocele, Nerve growth factor, Newborns, Neurotrophic factors.

Introduction

Myelomeningocele (MMC) is one of the most common neural tube defect that arises during embryonic development as a result of defective primary neurulation[1]. Common physical problems associated with MMC include motor deficits, urogenital and intestinal dysfunctions, skeletal malformations, and hydrocephalus. The aetiology of MMC is multifactorial and involves genetic and environmental variables[2]. The most compelling environmental risk factor is inadequate maternal folate status. It has been proved that folic acid plays a crucial role in preventing up to 70% of MMC cases[3]. Conversely, at least 30% of MMC cases are likely to result from mechanisms that are not potentially related by low folate levels suggesting that other factors may be implicated as risk factors, such as loss of amino acids from the fetus and increased activities of free radicals in the early stage of neural tube formation[4,5]. Recently, the accepted hypothesis of an intrinsic aetiology for the sensorimotor deficits in MMC has been challenged by experimental models of surgically induced MMC secondary to spinal cord injury (SCI)[6]. It has been suggested that direct trauma or inflammatory stimuli to the exposed fetal spinal cord might occur in utero, thereby, eliciting secondary damage to the spinal neural cells[6]. A support to this hypothesis has come from immunohistochemical investigations on experimental MMC, which demonstrated massive spinal cord astrocytosis and axonal loss at fetal stages[7,8]. Both astrocytosis and axonal loss are pathological features in MMC patients that can be studied and monitored by measuring some specific neurotrophic factors, such as brain

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derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), and nerve growth factor (NGF). These factors play a key role in the stimulation of sprouting, synaptic plasticity and reorganization after SCI and head injury, providing neuroprotection and enhancing some neuroregenerative activities[9-12]. NGF protects the damaged neurons by promoting up-regulation of doublecortin expression, and intraventricular NGF administration has been shown to improve cerebral blood flow in hypoxic-ischemic brain injury[13,14]. Acting on spinal cholinergic neurons NGF plays a key role also in bladder dysfunction following SCI for its action on neurochemical and electrophysiological reorganization of the micturition reflex[15]. Moreover, implanting NGF at the site of dorsal hemisection of the spinal cord improves the corticospinal axonal sprouts and bladder function after spinal cord damage[16]. All these experimental studies demonstrated the key role of this neurotrophin in neuronal survival and axonal regeneration after SCI, but there are no reports on endogenous changes of NGF in the CSF of newborns with MMC. Based on those previous experiences demonstrating the NGF role on neural cell differentiation and neuroprotection, the aim of this study was to investigate the expression of NGF in the CSF of newborns with MMC to determine its correlation with this malformation.

Patients and Methods

This study evaluated the expression of NGF in the CSF of newborns affected by MMC and admitted to our Institution between January 2005 and January 2012. All these infants were born at term from non consanguineous parents, after uncomplicated pregnancy and with normal birth weight. All of them underwent the neurosurgical correction of the malformation within 24 h from the birth. As controls, CSF samples collected in the same period from newborns who underwent lumbar puncture to rule out meningitis were used. Patients and control subjects were matched for age, sex and weight, respectively. To measure the levels of NGF, we collected CSF samples withdrawn from the myelomeningocelic sac immediately before starting the neurosurgical operation. All patients were not under any medications that could affect the NGF expression. All CSF samples were centrifuged for 10 minutes at 5000 rpm, and the supernatant was immediately stored at -70°C until analysis. The study was approved by the Ethical Committee of the Hospital. The parents of all patients involved provided written informed consent.

NGF Assays

NGF was quantified using a two-site immunoassay kit from Promega Corporation (Madison, WI, USA). 96-well plates were coated with 100 (L/well of monoclonal anti-NGF antibody. After overnight incubation at 4°C, the antibody was removed from the plates and the samples were incubated in coated wells (100 (L/well) for 6 hours at room temperature. The plates were then washed 5 times with buffer [0.05 M carbonate buffer (pH 9.5), 1% BSA (bovine serum albumin)] and the antigen was incubated overnight with polyclonal anti-human NGF antibody at 4°C. The plates were washed again with buffer [0.05 M carbonate buffer (pH 9.5), 1% BSA] and incubated with anti-chicken IgY HRP (horseradish peroxidase-conjugated) conjugate for 2 hours at room temperature. The plates were incubated with a tetramethylbenzidine (TMB)/peroxidase substrate solution for 15 minutes, and 1 M phosphoric acid was added (100 (l/well). The colorimetric reaction product was measured at 450 nm. NGF concentrations were interpolated from a NGF standard curve ranging from 15.6 to 1,000 pg/ml of purified human NGF. The sensitivity of this assay was 3 pg/ml and cross-reactivity with other related neurotrophins was less than 5%. All assays were performed in triplicate and NGF concentration was expressed as pg/mL.

Statistical Analysis

Statistical analysis of the data was performed using StatSoft (Tulsa, OK, USA) package considering the experimental conditions as a main factor. Analysis of variance was performed using the Tukey-Kramer test. The non-parametric Mann-Whitney two-tailed, two-sample test was used to perform statistical comparisons between children with MMC and the control group. Because the study population was small, we did not perform multivariate analyses to adjust for the effect of each parameter in the presence of the others. Spearman correlation coefficients were used to analyze the correlations between NGF levels, age, and clinical findings of the patients. A p-value of 0.05 was considered significant.

Results

Between January 2005 and January 2012, 29 newborns with MMC were born in our Hospital and were admitted to our Pediatric and Neurosurgical Departments for the treatment of their malformation. 14 of them (6 males and 8 females) were included in this study since they showed an unruptured myelomeningocelic sac that allowed the analysis of uncontaminated CSF. All patients underwent neurosurgical operation within 24 hours from the birth. The control group consisted of 14 newborns who underwent lumbar puncture to rule out meningitis. None of the controls had evidence of meningitis (as determined by negative cultures, chemical analysis and lack of pleocytosis). Table I reports the clinical and demographic characteristics of the MMC newborns together with the levels of NGF in their CSF. In newborns with MMC the NGF levels were significantly higher than in controls: NGF 63.05 ± 7.3 respect to 18.32 ± 4.5 pg/mL ($p < 0.001$) (Figure 1). On the contrary, no correlation was found between NGF expression in the CSF and different types of MMC malformation, such as the level of spinal lesion and the association with Chiari II syndrome (69.5 ± 5.5 vs 60.1± 6.5 pg/mL and 64.5 ± 5.0 vs 61.9 ± 5.5 pg/mL, respectively) (Figures 2 and 3). Due to the age of infants it was not possible to establish any correlation between NGF expression and motor and bladder dysfunction of patients.

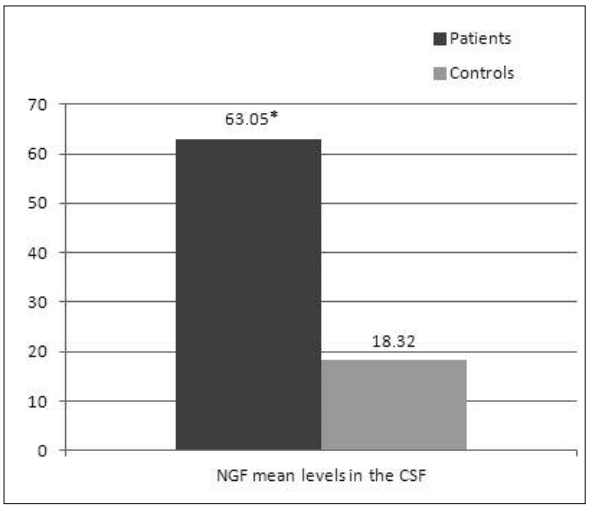

Figure 1. NGF levels in the CSF of newborns with myelomeningocele and in controls. Data represent mean value (± S.D.) in pg/mL and asterisk indicates significant difference among experimental groups ($p < 0.001$).

Discussion

Our study demonstrates a significant increase of NGF levels in the CSF of newborns with MMC compared with controls. Although we found no correlation between NGF expression and different types of MMC malformation, the observed NGF up-regulation in infants with MMC may represent a reactive response to the loss and damage of astrocytes and other neural cells at the site of MMC lesion, resulting in an increased biosynthesis of this neurotrophin in the CSF. In experimental animal models of SCI and MMC, a moderate recovery can be found after the spinal lesion. This recovery can be partly attributed to sprouting of spared and injured axons, rostral and caudal to the lesion, due to the neuroprotective action of neurotrophins[17]. NGF is

Table I. Clinical and demographic characteristics of newborns with myelomeningocele together with NGF levels in the CSF.

Patients	Sex	Type of lesion	Level of the lesion	Hydrocephalus	Chiari II-malformation	Time of surgery	NGF (pg/mL)
1	M	MMC	Lumbosacral	Yes	Yes	Within 24 h	80.5
2	M	MMC	Lumbosacral	No	No	Within 24 h	62.5
3	F	MMC	Lumbosacral	No	No	Within 24 h	68.5
4	F	MMC	Lumbosacral	No	No	Within 24 h	55.5
5	M	MMC	Lumbosacral	Yes	Yes	Within 24 h	60.0
6	F	MMC	Lumbosacral	No	No	Within 24 h	75.5
7	M	MMC	Lumbosacral	Yes	Yes	Within 24 h	52.2
8	M	MMC	Lumbosacral	Yes	Yes	Within 24 h	63.5
9	F	MMC	Lumbosacral	Yes	Yes	Within 24 h	74.5
10	F	MMC	Lumbosacral	No	No	Within 24 h	41.5
11	F	MMC	Lumbosacral	Yes	Yes	Within 24 h	56.5
12	M	MMC	Lumbosacral	No	No	Within 24 h	78.0
13	F	MMC	Lumbosacral	No	No	Within 24 h	49.0
14	F	MMC	Lumbosacral	No	No	Within 24 h	65.0

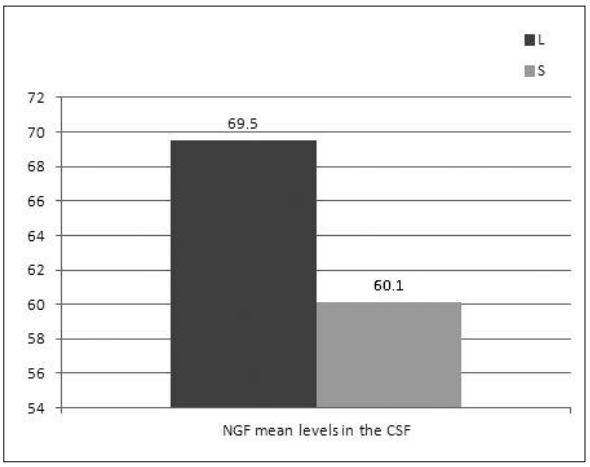

Figure 2. NGF levels in the CSF of newborns with lumbar (L) and sacral (S) MMC lesion. Data represent mean value (± SD) in pg/mL.

known to be one of the powerful survival factors for spinal neurons and several studies have shown the increase of this factor in experimental model of SCI, head trauma and meningoencephalitis[6,18]. Similarly, animals receiving NGF and other neurotrophins at the cell bodies of injured spinal neurons showed a significant increase in collateral sprouting and in the number of contacts with propriospinal interneurons that correlated significantly with the functional recovery[17,19]. In addition, local infusion of NGF, early after SCI, significantly improved motor function and reduced edema formation and nerve cell, glial cell, and axonal damage, stimulating the neuroprotective mechanisms at the level of the spinal cord injury[16]. Moreover, the transplantation with NGF-human umbilical cord blood cells into the spinal cord injured rat showed a positive effects on axonal regeneration with associated motor and autonomic improvement[12,20,21]. NGF is also involved in the pathogenesis of lower urinary tract disease, especially in conditions with altered neural function, such as neurogenic bladder[22]. This neurotrophin stimulates neural plasticity and bladder function and these activities are related to its properties on altering the sodium and potassium channels in bladder afferent fibers causing the alteration of the micturition reflex[22]. Our results are in keeping with these previous experimental findings confirming that the up-regulation of this neurotrophin participates in the pathophysiology of spinal cord injury in MMC malformation. Although we have analyzed only 14 newborns with MMC, the expression of NGF in their CSF doesn't seem to correlate with the severity of clinical manifestations and with the kind of spinal lesion, because NGF expression was not associated neither with the presence of Chiari II malformation and nor with the level of spinal lesion in these patients. Further researches, which include a larger number of patients, will be necessary to confirm our observation and to possibly establish if NGF determination in the CSF of infants with MMC may be an useful marker both of the severity of spinal cord damage and also for the motor and bladder dysfunction of the patients. Moreover, the finding of an early increase of NGF in the CSF of newborns with MMC suggests that this neurotrophin can be considered a molecular marker of spinal cord damage and could be useful as a specific diagnostic tool for the severity of MMC lesion and to shed light on the molecular pathogenesis of MMC and other human spinal cord disorders. The effect of NGF in the morphogenesis of neural tube occurs in very early stage of life by promoting the growth and the differentiation of neuroectoblasts and endoblasts[20,23]. The physiopathological role of neuronal cells in the developing of MMC damage has not been investigated previ-

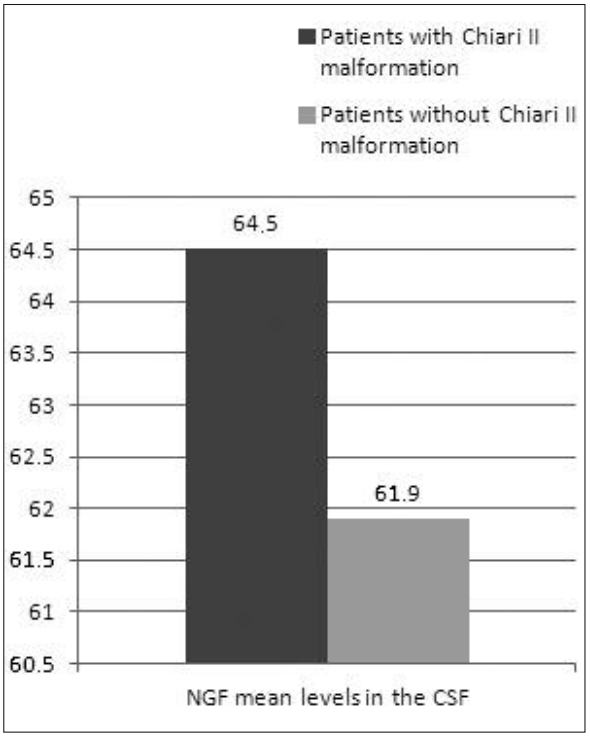

Figure 3. NGF levels in the CSF of newborns with or without Chiari II malformation. Data represent mean value (± SD) in pg/mL

ously, but there are some experimental data showing an astrocytic hypertrophy observed after axonal injury in different spinal cord damage, such as MMC and multiple sclerosis (MS), in which increased levels of neurotrophins are reported and related to motor disability and dysautonomyc dysfunction of the patients[24,25,26].

Conclusions

According to those observations, our results suggest that NGF determination in the CSF of newborns with MMC might be used as an index of axonal damage during the development of spinal lesion. NGF up-regulation might be an expression of functional mechanism aimed at stimulate axonal sprouting and synaptic reorganization of the damaged neural cells at the site of spinal cord injury. Although additional prospective and long-term studies are needed for a better understanding of neurotrophin pattern modulation in newborns with MMC, we believe that NGF and other neurotrophic factors may have promising clinical applications in these patients.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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