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Transplantation of umbilical cord blood mononuclear cells increases levels of nerve growth factor in the cerebrospinal fluid of patients with autism

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ABSTRACT. We aimed to evaluate the levels of growth factors in the cerebrospinal fluid (CSF) of patients with autism after transplantation of umbilical cord blood mononuclear cells (CBMNCs). Fourteen subjects diagnosed with autism received transplantation of CBMNCs first through intravenous infusion, and three times subsequently through intrathecal injections. A 2-mL sample of CSF was taken before each intrathecal injection. CSF levels of nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) were determined by enzyme-linked immunosorbent assay. All data are reported as means ± SD and were analyzed using the SPSS 10.0 software. One-way analysis of variance with *post-hoc* F-and Q-tests were performed for comparisons. NGF levels in the CSF were significantly increased after transplantation (213.54 ± 56.38 after the third versus 28.32 ± 12.22 ng/L after the first transplantation; $P < 0.05$), while VEGF and bFGF levels did

not change significantly. Therefore, transplantation of CBMNCs could increase NGF levels in the CSF of patients with autism.

Key words: Umbilical cord blood mononuclear cells; Growth factors; Cerebrospinal fluid

INTRODUCTION

Autism is a neurodevelopmental disorder characterized by severely impaired social interaction and communication skills, as well as restricted, repetitive, or stereotypical behavior. The prevalence of autism is estimated to be about five per 10,000 people worldwide. Diagnosis of autism relies mainly on behavioral criteria (Baird et al., 2003). Its symptoms often appear between 12 and 18 months of age, but diagnosis can only be made between the ages of 24 and 36 months (Mitchell et al., 2006). Moreover, some patients can be diagnosed with autism only in adulthood (Filipek et al., 1999). The exact causes of autism remain unclear, although it is generally accepted that autism may result from a combination of genetic and environmental factors. For example, the risk of autism is associated with prenatal viral infections, certain environmental agents, and autoimmune diseases (Newschaffer et al., 20

The characteristics of autism, such as impaired social development, lack of communication, and repetitive behaviors, greatly affect the normal development of patients and present a huge burden on patients' families. Current therapeutic approaches of autism include educational and behavioral interventions and medications, which are designed to meet the specific needs of the individual. However, no effective cure for autism has been reported to date. Recent evidence reveals several biomarkers associated with autism, such as oxidative stress, decreased methylation capacity, limited production of glutathione, and mitochondrial dysfunction, which may be utilized for the diagnosis and therapy of autism (Bradstreet et al., 2010).

Stem cells are highly proliferative, self-renewing, and multi-potent cells with the potential to treat human degenerative diseases. In recent years, stem cell therapy has shown promise in the treatment of various human diseases. For example, stem cells from bone marrow and umbilical cord blood have been used to treat leukemia (Park and Lee, 2013). Umbilical cord-derived mesenchymal stem cells (UCMSCs) from humans are abundant, self-renewing multi-potent stem cells with a differentiation potential towards neural lineages and the ability to secrete growth and neurotrophic factors (Neuss et al., 2004; Kuan and Barker, 2005). We recently reported that transplantation of umbilical cord blood mononuclear cells (CBMNCs) and UCMSCs showed therapeutic efficacy in patients with autism based on the index of Childhood Autism Rating Scale (CARS), Clinical Global Impression (CGI) scale, and Aberrant Behavior Checklist (ABC) (Lü et al., 2013). However, the underlying mechanism remained elusive. In this study, we investigated the changes in the levels of nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) in the cerebrospinal fluid (CSF) of patients with autism after transplantation of CBMNCs.

MATERIAL AND METHODS

Study subjects

The study protocol and consent forms were approved by the Institutional Review

Board of Shandong Jiaotong Hospital under the auspices of the National Ministry of Health. Subjects were recruited from Shandong Jiaotong Hospital between January 2009 and December 2010. Eligible subjects in this study included 12 boys and 2 girls (3-12 years old) diagnosed with autism, in accordance with the diagnostic criteria for autism in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (Filipek et al., 1999; Mitchell et al., 2006), and with a Childhood Autism Rating Scale (CARS) score ≥ 30. The study protocols were approved by the Ethics Committee of Shandong Jiaotong Hospital and guardians of all subjects signed informed consent forms. Exclusion criteria included: 1) prior history of severe allergic reactions; 2) any severe psychiatric disorder; 3) seizures within the past 6 months; 4) autism caused by active epilepsy, cerebrovascular diseases, or brain trauma; 5) Severity of illness (SI) of Clinical Global Impression (CGI) scale evaluated as "normal" or "borderline mentally ill" or "mildly ill"; 6) moderate or severe extrapyramidal symptoms or tardive dyskinesia; 7) severe self-injury behavior; 8) active systemic or severe focal infections such as human immunodeficiency virus (HIV) and hepatitis; 9) autoimmune diseases; 10) severe pulmonary and hematological diseases, malignancy, or hypoimmunity; 11) other treatments that could affect the safety, efficacy, and/or evaluation of stem cell therapy.

Cell preparation and transplantation

The CBMNCs were provided by Shenzhen Beilai Biotechnology Co., Ltd. Fresh human cord blood was obtained from informed healthy donors in accordance with the sterile procurement guidelines established by the hospital in conjunction with the National Ministry of Health. After collection, each sample was tested for communicable diseases, including hepatitis B, hepatitis C, HIV, cytomegalovirus, and syphilis, as well as the enzyme alanine aminotransferase, and then transferred for cell preparation in the GMP laboratories.

Cord blood was diluted with saline (1:1) and 30 mL of the diluted blood was then added to 15 mL Ficoll and centrifuged (700 g x 22 min). Mononuclear cells were collected and washed twice in saline. Contaminating erythrocytes were lysed with lysis buffer comprised of injection grade water. Cell density was adjusted to 2-6 x 10^6/mL and seeded in Dulbecco's modified Eagle's medium/nutrient mixture F-12 culture medium with bFGF and epidermal growth factor at a concentration of 20 ng/mL. Culture media was mixed with 2% v/v B-27 Stem Cell Culture Supplement. Cells were cultured at 37°C with saturated humidity and 5% CO_2 by volume and harvested for clinical application after 4-7 days of cultivation. The final CBMNC product contained 0.2-1.0% $CD34^+$ cells as determined by flow cytometry.

After extensive discussion answering all questions, written informed consent was obtained from each subject's guardian before initiating the scheduled treatments. The subjects received four cell transplantations at 7-day intervals. Approximately 2 x 10^6/kg body weight CBMNCs were infused with normal saline intravenously (20 mL) and/or intrathecally (2 mL) for each treatment. The subjects received the first transplantation through intravenous infusion and three subsequent transplantations through intrathecal injections.

Enzyme-linked immunosorbent assay (ELISA)

A 2 mL CSF sample was collected from each subject before each intrathecal injection. The samples were centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was aliquoted

and stored at -70°C. The levels of NGF, VEGF, and bFGF in the supernatant were determined using an ELISA kit (USCN Life Sciences Inc., Wuhan, China) according to manufacturer instructions.

Safety and efficacy measures

Treatment safety was evaluated with documentation of physical examination, vital signs, and adverse events; complete blood count, liver and renal function, serum glucose, lipid profile, and immunology testing including immunoglobulin (Ig) A/G/M and complement C3/C4 and T-cell subsets at baseline (pre-treatment) and 4, 8, and 24 weeks after the first cell transplantation. Subjects were assessed using the CARS at baseline and 4, 8, 16, and 24 weeks after the first cell transplantation for efficacy. All assessments were conducted by physicians from the Shandong Mental Health Center.

Statistical analysis

All data are reported as means ± SD and were analyzed using the SPSS 10.0 software. One-way analysis of variance (ANOVA) with *post-hoc* F- and Q tests were performed for comparisons. A P value less than 0.05 was considered statistically significant.

RESULTS

ELISA was used to measure the levels of NGF, VEGF, and bFGF in the CSF of 14 subjects with autism before and after CBMSCs transplantation (Table 1).

Table 1. Levels of NGF, VEGF, and bFGF in the CSF of subjects (means ± SD, N = 14).

Time	NGF (ng/L)	VEGF (ng/L)	bFGF (ng/L)
1 week after first treatment	28.32 ± 12.22	31.93 ± 14.63	23.43 ± 15.92
1 week after second treatment	33.17 ± 14.56	36.69 ± 12.57	28.67 ± 12.13
1 week after third treatment	213.54 ± 56.38[a]	43.62 ± 12.55	35.33 ± 11.25

[a]P< 0.05 *vs* 1 week after first treatment.

NGF levels in the CSF were significantly increased 1 week after the third transplantation (213.54 ± 56.38 ng/L) compared to 1 week after the first treatment (28.32 ± 12.22 ng/L, P < 0.05). NGF levels in the CSF were increased 1 week after the second transplantation (33.17 ± 14.56 ng/L) compared to 1 week after the first treatment (28.32 ± 12.22 ng/L), but this difference was not significant (P > 0.05). There were no significant changes in VEGF and bFGF levels in the CSF after transplantation (P > 0.05).

Treatment safety was evaluated by complete blood count, liver and renal function tests, serum glucose, lipid profile, and immunology tests including immunoglobulin (Ig) A/G/M, complement C3/C4 and T-cell subsets, and tests for HIV, syphilis, and HBV at baseline (pre-treatment) and 4, 8, and 24 weeks after the first transplantation. There were no significant changes in these indices for all subjects. There were two cases of low-grade fever, which subsided without medical intervention.

Childhood autism rating scale (CARS)

The total scores obtained following CARS assessment decreased from 46.43 ± 8.65 at baseline to 37.14 ± 10.15 at 24 weeks. In addition there were significant differences in CARS scores at 4, 8, and 16 weeks compared with baseline (Table 2).

Table 2. CARS total score.

Baseline	4 weeks	8 weeks	16 weeks	24 weeks
46.43 ± 8.65	39.21 ± 8.63	36.64 ± 7.07	35.14 ± 7.77	37.14 + 10.15

DISCUSSION

It had long been assumed that neurons have limited ability to regenerate. However, the discovery of neural stem cells brought with it the promise of new therapeutic approaches for the treatment of diseases of the central nervous system. As one potential source of neural stem cells, CBMNCs contain various progenitor cells including a large population of $CD34^+$ hematopoietic stem cells, a small population of mesenchymal stem cells and very few endothelial progenitor cells and muscle satellite cells. These progenitor cells are highly proliferative with the potential to differentiate towards neural cell fate and secrete various growth and neurotrophic factors (Fan et al., 2005; Habich et al., 2006; Chen et al., 2005, 2007; Lee et al., 2007; Bachstetter et al., 2008). These factors when secreted into the immediate extracellular environment, play important roles in paracrine signaling to regulate hematopoietic processes, immune responses, angiogenesis, and cell proliferation, differentiation and apoptosis. Furthermore, CBMNCs have been shown to promote endogenous neural cell regeneration (McGuckin et al., 2008), and preclinical studies have demonstrated their potential in treating diseases of the nervous system.

Mesenchymal stem cells (MSCs) are multi-potent stem cells derived from mesoderm and have been found in bone marrow, adipose tissue, umbilical cord tissue, and placenta. MSCs secrete neurotrophic factors and, when transplanted, can promote the secretory activity of host cells. Neurotrophic factors regulate neuronal proliferation and differentiation, and provide support and nutrients to neurons. Transplantation of MSCs promoted the production of brain-derived neurotrophic factor (BDNF) and NGF, both of which are required for neuronal survival, differentiation, and myelination (Chang et al., 2011; Lopatina et al., 2011). Several mechanisms have been proposed to explain why transplanted MSCs restore neuronal function. First, MSCs secrete neurotrophic factors. For example, MSCs cultured *in vitro* secrete BDNF and NGF into the culture media. Transplantation of MSCs increased the levels of these factors in injured areas of a middle cerebral artery occlusion (MCAO) mouse model and in the CSF of a traumatic brain injury model (Facchiano et al., 2002; Walker et al., 2009). Second, MSCs promote the self-restoration of host cells. MSCs regulate the cellular microenvironment by secreting growth factors and promoting the production of neurotrophic factors by glial cells, which may promote progenitor/precursor proliferation, migration, and differentiation in the adult brain. For example, injection of bFGF and NGF into the brain promotes the proliferation, migration, and differentiation of progenitors/precursors (Bachstetter et al., 2008).

Neurotrophic factors reduce apoptosis in the penumbral zone, stimulate the proliferation of endogenous cells in the subventricular zone, and promote neuronal regeneration, dendrite formation, signal transduction, and the release of neurotransmitters (Lindholm, 1997; Li et al., 2002). Third, MSCs promote angiogenesis. It has been shown that after bone marrow stromal cell transplantation, the secretion of VEGF was promoted in the ischemic boundary zone after stroke (Chen et al., 2003).

Several studies have shown that cerebral hypoperfusion is associated with many core symptoms in autism (Freeman et al., 2004; Madduri et al., 2009). Generalized brain hypoperfusion, peaking in frontal and prefrontal regions, was observed in children with autism and associated with cognitive and neuropsychological defects (Freeman et al., 2004; Madduri et al., 2009). In addition, decreased cerebral perfusion, especially in the temporoparietal areas, has been linked to cognitive impairments, such as language deficits, impairment of cognitive development and object representation, and abnormal perception and responses to sensory stimuli (Freeman et al., 2004; Madduri et al., 2009). Inadequate perfusion resulting in brain tissue hypoxia not only caused neuronal apoptosis and necrosis, but also led to abnormal brain tissue metabolism and accumulation of pathological levels of neurotransmitter (Freeman et al., 2004; Madduri et al., 2009). Therapeutically targeting cerebral ischemia and resulting hypoxia may be an alternative therapeutic approach in autism (Freeman et al., 2004; Madduri et al., 2009). Therapeutic angiogenesis promoted by systemic administration of cord blood $CD34^+$ stem cells to overcome ischemia has been experimentally demonstrated *in vitro* and in animal models. It has been shown that endothelial progenitor cells, contained in the $CD34^+$ cell population enriched in CBMNCs, has the capacity to trigger angiogenesis in ischemic tissue (Freeman et al., 2004; Madduri et al., 2009). The circulating $CD34^+$ progenitors in the CBMNC population, having the potential for endothelial development, were recruited to injury sites and developed into new endothelial cells to either repair the injured endothelial wall or sprout new vascular structures (Freeman et al., 2004; Madduri et al., 2009). Moreover, human $CD34^+$ cells and hematopoietic precursors can secrete numerous angiogenic factors such, as vascular endothelial growth factor, HGF, and insulin-like growth factor-1 (Freeman et al., 2004; Madduri et al., 2009). CBMNC therapies have been successfully translated into preclinical applications for functional recovery in various ischemic animal models through the enhancement of angiogenesis around the site of degeneration (Freeman et al., 2004; Madduri et al., 2009). Given the potency of cord blood $CD34^+$ cells in promoting angiogenesis in ischemic areas, CBMNCs may be useful for the improvement of the cerebral hypoperfusion and hypoxia that has been suggested to occur in the brains of individuals with autism (Freeman et al., 2004; Madduri et al., 2009).

A variety of growth factors and neurotrophic factors regulate neuronal differentiation, survival, and regeneration. Among them, NGF provides support and nutrients to neurons, promotes neurite growth, and regulates neuronal differentiation, maturation, and regeneration in both the central and peripheral nervous systems (Freeman et al., 2004; Madduri et al., 2009). Consistent with these roles of NGF, in this study we observed significantly increased levels of NGF in the CSF of patients with autism after therapy using CBMNC transplantation. However, the levels of bFGF and VEGF were not significantly changed after transplantation; the reasons for this are unclear and need further investigation. Based on these data we postulate that increased levels of NGF may at least account for the therapeutic efficacy of CBMNC transplantation in patients with autism as we reported previously (Lü et al., 2013). Neverthe-

less, further studies are needed to reveal additional mechanisms by which transplantation of CBMNCs achieves therapeutic efficacy in autism.

Conflicts of interest

The authors declare no conflict of interest.

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