Mononuclear cells from the cord blood and granulocyte-colony stimulating factor-mobilized peripheral blood: is there a potential for treatment of cerebral palsy?

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Abstract
To investigate a possible therapeutic mechanism of cell therapy in the field of cerebral palsy using granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells (mPBMCs), we compared the expression of inflammatory cytokines and neurotrophic factors in PBMCs and mPBMCs from children with cerebral palsy to those from healthy adult donors and to cord blood mononuclear cells donated from healthy newborns. No significant differences in expression of neurotrophic factors were found between PBMCs and mPBMCs. However, in cerebral palsy children, the expression of interleukin-6 was significantly increased in mPBMCs as compared to PBMCs, and the expression of interleukin-3 was significantly decreased in mPBMCs as compared to PBMCs. In healthy adults, the expression levels of both interleukin-1β and interleukin-6 were significantly increased in mPBMCs as compared to PBMCs. The expression of brain-derived neurotrophic factors in mPBMC from cerebral palsy children was significantly higher than that in the cord blood or mPBMCs from healthy adults. The expression of G-CSF in mPBMCs from cerebral palsy children was comparable to that in the cord blood but significantly higher than that in mPBMCs from healthy adults. Lower expression of pro-inflammatory cytokines (interleukin-1β, interleukin-3, and -6) and higher expression of anti-inflammatory cytokines (interleukin-8 and interleukin-9) were observed from the cord blood and mPBMCs from cerebral palsy children rather than from healthy adults. These findings indicate that mPBMCs from cerebral palsy and cord blood mononuclear cells from healthy newborns have the potential to become seed cells for treatment of cerebral palsy.

Key Words: neurotrophic factors; inflammatory cytokines; cord blood; G-CSF mobilized peripheral blood; mononuclear cell; cerebral palsy; children; neural regeneration

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Introduction
Cerebral palsy is a group of chronic nonprogressive disorders characterized by aberrant posture and movements caused by abnormal brain development or injury. Cerebral palsy occurs in 1–3% of live newborns, and in high-risk babies such as those with very low birth weight, the incidence is increased to 8–40% (Bosanquet et al., 2013). The combined motor, sensory, cognitive and occupational impairments caused by cerebral palsy may lead to substantial social and economic burdens to the families, health care systems, and communities of these individuals. Currently, therapies for cerebral palsy patients are limited to supportive interventions (Ruff et al., 2013). Recently, stem cell therapies have been investigated as possible new treatment modalities for neuronal repair in young patients with cerebral palsy, and bone marrow- or cord blood (CB)-derived mesenchymal stem cells (MSCs) are the most commonly used sources of cellular therapy. The potential of mobilized peripheral blood mononuclear cells (mPBMCs) as a MSC source has also been suggested (Deng et al., 2011). Although not fully understood, the clinical effects of MSCs seem to stem from indirect paracrine effects rather than from direct cellular effects or neuronal regeneration (Seo et al., 2012). CB mononuclear cells (CB-MNCs) without any manipulation have been also used in place of MSCs. CB cell therapies can be considered an optimal stem cell source for regenerative medicine due to their potential to develop into any tissue in the body. In human clinical trials using autologous CB in cerebral palsy patients, improvement in gross motor function and neurological impairments without critical side effects have
been reported (Harris et al., 2009; Papadopoulos et al., 2011; Lee et al., 2012). CB-MNCs express neurotrophic factors and produce cytokines that play critical roles in repairing brain damages associated with cerebral palsy (Fan et al., 2005).

Granulocyte-colony stimulating factor (G-CSF) is widely used for the treatment of neutropenia and has also been used for hematopoietic stem cell mobilization in autologous and allogeneic transplantation without serious side effects (Pulsipher et al., 2006). In addition, it has been suggested that G-CSF is an endogenous ligand that counteracts programmed cell death and precipitates neurogenesis (Schneider et al., 2005). These functions of G-CSF in the central nervous system could be explained by autocrine signaling by neuroprotective factors such as brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), and erythropoietin (Kokaia et al., 1993; Shingo et al., 2001; Ogunshola et al., 2002). Therefore, G-CSF could be safely used for treating cerebral palsy in children when administered to mobilize bone marrow stem cells to the peripheral circulation, making mobilized peripheral blood stem cells, that is, mPBMCs a possible alternative source of cellular therapy. In a previous study, we performed a clinical trial for mPBMC collections from cerebral palsy patients; our data supported the safety and feasibility of mPBMCs isolated from cerebral palsy children (Moon et al., 2013).

While previous studies have investigated the cytokines and/or neurotrophic factors of MSCs derived from CB or mPBMCs (Urdzikova et al., 2006; Zhang et al., 2011), and pro- and anti-inflammatory cytokines may have a large impact on the neurological outcome of patients (Moghaddam et al., 2015), to our knowledge there have been no comparative studies between MNC of CB and mPBMCs. To investigate possible therapeutic treatments using mPBMCs for cell therapy in the field of neurological disorders and also to reveal the possible role of G-CSF for neuroprotection, we compared the expression of inflammatory cytokines and neurotrophic factors in PBMCs (PBMCs means circulating mononuclear cells in the peripheral blood before G-CSF injection) and mPBMCs from cerebral palsy children and healthy adult donors and in the CBs donated from healthy newborns.

**Materials and Methods**

**Sample preparation and study design**

CB, which had been cryopreserved for research use as monocellular cell fractions after depletion of red blood cells and plasma by density gradient method, was supplied from the Public Cord Blood Bank at Cha University Hospital, Seoul, Republic of Korea. The CB fulfilled the criteria for research use as defined by the Cord Blood Management and Research Act, Republic of Korea (Lee, 2010).

Samples from healthy adults were obtained from volunteer donors who donated their peripheral blood stem cells after informed consent and permission from the Korea Marrow Donor Program. Blood samples from cerebral palsy children were obtained from participants in a clinical research trial involving mPBMC therapy for cerebral palsy children, which was approved by the Institutional Review Board of Hanyang University Hospital (HYUH IRB 2011-C-21) in the Republic of Korea and in compliance with the World Medical Association outlined in the Declaration of Helsinki. PBMCs were separated from the peripheral venous blood using a Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient method (Fuss et al., 2009) and collected via a central venous catheter prior to apheresis from 14 cerebral palsy children and 14 healthy adult volunteers.

mPBMCs were harvested from the apheresed products using a blood cell separator (CS3000®, Baxter Healthcare Corp., Deerfield, IL, USA) on the 5th day after 5 consecutive days of 10 μg of intravenous or subcutaneous G-CSF (Leucostim®, Dong-a ST, Seoul, Korea) treatment. Thereafter, mPBMCs were prepared by red blood cell lysis using lysis buffer (BD, San Diego, CA, USA) from an aliquot of apheresed products. Separated PBMCs and mPBMCs were cryopreserved at −196°C for over 3 months and then analyzed after thawing.

We compared the intracellular expression of five neurotrophic factors (BDNF, gial cell-derived neurotrophic factor [GDNF], G-CSF, VEGF, insulin like growth factor [IGF]-1) and seven inflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-2, IL-3, IL-6, IL-8, IL-9) with flow cytometry analysis from each sample. This study was approved by the Institutional Review Board of Hanyang University (HYI-11-013-1).

**Total nucleated cell (TNC)/CD34+ cell count and viability**

The TNC count was measured using a Sysmex K-800 (Sysmex Corporation, Kobe, Japan) automated cell counter. For CD34+ cell count, isolated MNCs were stained with CD34 antibodies and analyzed with Lysys II software flow cytometry (BD, San Jose, CA, USA). Cell viability of pre-freezing and post-thawing was measured by trypan blue staining (Xiao et al., 2003).

**Intracellular staining**

Cells were stimulated to express cytokines by 100 ng/mL lipopolysaccharide or 50 ng/mL phorbol 12-myristate 13-acetate and 1 μg/mL ionomycin for 6 or 24 hours. In order to accumulate the cytokines within the cells, protein secretion needed to be inhibited by addition of protein secretion-inhibiting reagents during the stimulation. Therefore, cells were cultured in a 37°C CO2 incubator with 0.667 μL per well of Becton-Dickinson (Franklin Lakes, NJ) golgistop protein transport inhibitor (containing monensin). After incubation, cells were transferred to a 5 mL polystyrene round bottom tube, and 250 μL of fixation/permeabilization solution was added to each well and incubated for 20 minutes at 4°C. Harvested cells were washed twice with 1 mL of 1X BD Perm/Wash buffer and centrifuged at 100 × g at 20°C for 5 minutes. After removing the supernatant, fixed/permeabilized cells were resuspended in 200 μL of BD Perm/Wash buffer containing a PE-conjugated antibody (BDNF, GDNF, G-CSF, VEGF, IGF-1, TNF-α, IL-1β, IL-2, IL-3, IL-6, IL-8, IL-9) at pre-determined optical concentrations and appropriate isotype control, and incubated at 4°C for 30 minutes in the
Flow cytometry analysis

Flow cytometry was used to divide the cell population by relative size and either relative granularity or internal complexity into four fractions: P1, P2, P3 and P10 (Figure 1). Since a large number of dead cells were observed in the P1 and P3 fractions by staining with propidium iodide, they were gated out, and only the P2 and P10 fractions were analyzed. Samples were run using a FACS Canto II (BD) with FACS Diva Software (BD, Franklin Lakes, NJ, USA) that was set to acquire 10,000 events in a tight side scatter and forward scatter. The expression of cytokines was determined by the percentage of positive stains of each monoclonal antibody. The degree of auto-fluorescence and non-specific binding of antibodies was determined with an isotype control (Freer et al., 2013).
Figure 3 Differences in cytokine expression in mPBMCs and PBMCs from healthy adults.
The expression of IL-1β (P = 0.048) and IL-6 (P = 0.006) was significantly increased in mPBMCs (n = 14) than in PBMCs (n = 14). The Wilcoxon signed-rank test, Kruskal-Wallis test and Mann Whitney U test were used for intergroup comparisons. All statistical analyses were conducted using IBM SPSS software. The data were expressed as the mean ± SD and *P < 0.05. BDNF: Brain-derived neurotrophic factor; GDNF: glial cell-derived neurotrophic factor; G-CSF: granulocyte-colony stimulating factor; VEGF: vascular endothelial growth factor; IGF-1: insulin-like growth factor-1; TNF-α: tumor necrosis factor alpha; IL: interleukin; PBMC: peripheral blood mononuclear cell; mPBMC: mobilized peripheral blood mononuclear cell.

Figure 4 Cytokine profiles of CB and mPBMCs in CP children versus healthy adults.
The expression of BDNF was significantly increased in mPBMCs (n = 14) from CP children as compared to either mPBMCs (n = 14) from healthy adults (P = 0.027) or CBs (n = 14, P = 0.035). The expression of G-CSF was significantly increased in mPBMCs from CP children as compared to mPBMCs from healthy adults (P = 0.001) and was significantly increased in CBs as compared to mPBMCs of healthy adults (P = 0.002). The expression of IL-1β was significantly increased in mPBMCs from healthy adults as compared to mPBMCs of CP children (P = 0.001) and in the CB (P < 0.0001). The expression of IL-8 was significantly increased in mPBMCs from CP children as compared to that in healthy adults (P = 0.012) and was also significantly increased in the CB as compared to mPBMCs from healthy adults (P = 0.044). The expression levels of IL-3 (P = 0.004) and IL-6 (P = 0.031) were significantly increased in mPBMCs of healthy adults as compared to those in the CB. IL-9 was significantly increased in mPBMCs from the CB as compared to that in healthy adults (P = 0.014). The Wilcoxon signed-rank test, Kruskal-Wallis test and Mann Whitney U test were used for intergroup comparisons. All statistical analyses were conducted using IBM SPSS software. The data were expressed as the mean ± SD and *P < 0.05. BDNF: Brain-derived neurotrophic factor; GDNF: glial cell-derived neurotrophic factor; G-CSF: granulocyte-colony stimulating factor; VEGF: vascular endothelial growth factor; IGF-1: insulin-like growth factor-1; TNF-α: tumor necrosis factor alpha; IL: interleukin; PBMC: peripheral blood mononuclear cell; mPBMC: mobilized peripheral blood mononuclear cell; CP: cerebral palsy; CB: cord blood.
Statistical analysis
Each value was described as the median value with standard deviation and range. The Wilcoxon signed-rank test, Kruskal-Wallis test and Mann-Whitney test were used for intergroup comparisons. All statistical analyses were conducted using IBM SPSS software (version 21; IBM Co., Armonk, NY, USA). 

Results

Number and viability of PBMCs, mPBMCs and CB-MNCs
We compared the number and viability just between PBMCs and mPBMCs because the comparison of the number of CB-MNCs and PBMCs or mPBMCs was not appropriate. The median number of TNC and their viabilities in PBMCs in cerebral palsy children were comparable to those of healthy adults. However, TNC count \( (P = 0.001) \) and CD34+ cell count \( (P = 0.002) \) of mPBMCs were significantly higher in healthy adults than in cerebral palsy children (Table 1). Conversely, mPBMC viabilities before freezing \( (P = 0.001) \) and after thawing \( (P = 0.003) \) were higher in mPBMC from cerebral palsy children than in those from healthy adults (Table 1).

Differences in cytokine expression between mPBMCs and PBMCs in children with cerebral palsy and in healthy adults
No significant differences in the expression of neurotrophic factors were found between PBMCs and mPBMCs. However, in cerebral palsy children, the expression of IL-6 was increased in mPBMCs over PBMCs \( (P = 0.035) \), and IL-3 was significantly decreased in mPBMCs as compared to PBMCs \( (P = 0.048) \) (Figure 2). In healthy adults, the expression levels of both IL-1β \( (P = 0.048) \) and IL-6 \( (P = 0.006) \) were significantly increased in mPBMCs as compared to PBMCs (Figure 3).

Comparison of cytokine profiles between CB and mPBMCs of cerebral palsy children and healthy adults
The expressions of most cytokines in mPBMCs of cerebral palsy children were comparable to those in healthy donated CBs and adult volunteers (Figure 4). However, the expression of BDNF was significantly increased in mPBMCs from cerebral palsy children as compared to either mPBMCs from healthy adults \( (P = 0.027) \) or CBs \( (P = 0.035) \). The expression of G-CSF was significantly increased in mPBMCs from cerebral palsy children as compared to mPBMCs from healthy adults \( (P = 0.001) \) and was significantly decreased in the CB as compared to mPBMCs of healthy adults \( (P = 0.002) \). The expression of IL-1β was significantly increased in mPBMCs from healthy adults as compared to mPBMCs of cerebral palsy children \( (P = 0.001) \) and in the CB \( (P < 0.0001) \). The expression of IL-8 was significantly increased in mPBMCs from cerebral palsy children as compared to that in healthy adults \( (P = 0.012) \) and was also significantly increased in the CB as compared to mPBMC from healthy adults \( (P = 0.044) \). The expression levels of IL-3 \( (P = 0.004) \) and IL-6 \( (P = 0.031) \) were significantly increased in mPBMCs of healthy adults as compared to those in the CB. IL-9 was significantly increased in mPBMCs from the CB as compared to that in healthy adults \( (P = 0.014) \).

Discussion
Stem cell therapy has been proven effective for improving neuronal recovery in both animal models and human trials (Chicha et al., 2013). Among a series of stem cell sources used to repair neurological diseases, intravenous administration of autologous CB has been used to try to counteract neurological injuries and impairments. CB-MNCs are a rich source of stem cells and are easy to obtain by noninvasive procedures. It induces neurotrophic factor production, which may remove abnormal synapses from damaged neurons and guide the formation of newly formed synapses (Morgan et al., 2004).

Because autologous CB is limited in supply, the clinical usage of CB is restricted. However, we suggest that mPBMCs could potentially be used for treating neurological impairments. Pettengell et al. (1994) suggested that stem cells from the CB and leukapheresis products are comparable in long-term culture-initiating cells. Tondreau et al. (2005) assessed the potential of mobilized peripheral blood and CB as a source of MSCs. In addition, it would be possible to perform a clinical trial of mPBMC treatment in cerebral palsy children, since we have already observed the safety of administering G-CSF and collecting mPBMCs in cerebral palsy children (Moon et al., 2013).

It remains unresolved whether stem cells have the ability to pass across the blood-brain barrier and migrate to targeted brain lesions. In vivo, stem cells possess certain molecular mechanisms involving adhesion molecules, chemokines, and proteases, which enable transmigration of stem cells into the brain (Liu et al., 2013). Although inflammation-induced blood-brain barrier disruption and increased permeability can result in developmental damage to the brain in early human life, it is assumed that proinflammatory cytokines expressed by infused stem cells may affect the junctional structures at the blood-brain barrier and leave a way open for migration of stem cells from the circulatory blood; some of these stem cells may differentiate into the microglia in the brain (Stopl et al., 2009). Microglia can then release neurotrophic factors involved in regeneration of the brain. Neurotrophins and cytokines are co-expressed at the location of neuronal injury. The interactions of these factors modulate both neuronal degeneration and regeneration (Otten et al., 2000).

The expression and roles of neurotrophic factors and cytokines in transplanted cells have not been fully elucidated, nor has their expression in cerebral palsy children been investigated. In this study, we analyzed neurotrophic factors and cytokines expressed in mPBMCs from the cerebral palsy children as compared with those found in healthy adults and donated CBs. Among the neurotrophic factors used in this study, BDNF is known as a factor that regulates neuronal development and function (Allen et al., 2013). Overexpressing BDNF in gene-modified human bone marrow stem cells further increases the potential therapeutic
Cytokines are secreted from cells and function as communicators between cells in both paracrine and endocrine fashions. They mediate inflammatory responses and are also important for the repair and defense of neuronal tissues following trauma (Lin et al., 2013). Well-studied cytokines include TNF-α, IL-1 family (IL-1α, IL-1β, IL-18), IL-6, and IL-10. TNF-α is a classic pro-inflammatory factor and stimulates macrophages, monocytes and NK cells. Increased concentrations of TNF-α after diffuse axonal injury in head trauma imply that TNF-α participates in secondary neuronal injury (Ciāllella et al., 2002; Campbell et al., 2007; Lin et al., 2013). However, even pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α have both deleterious and beneficial effects on neuronal cells (Winter et al., 2004). Their roles in stem cell therapy for neuronal regenerative treatment should be further investigated. IL-2 is an essential factor for immune homeostasis, normal regulatory T cell function, and self-tolerance in the immune system. Brain-derived IL-2 plays an essential role in the maintenance of septohippocampal projection neurons in vivo (Meola et al., 2013). IL-3, expressed in hematopoietic and nonhematopoietic cells, is an important regulator that exhibits pleiotropic activities. The major roles of IL-3 are increasing the activity of Bcl-2, activating neuroprotection, and preventing apoptosis (Rojo et al., 2008). We have limited research data to provide a concrete base of changes in IL-6 expression. We need further research on that matter. IL-6 has been recognized as an important pro-inflammatory cytokine secreted by leukocytes and activated glia in the nervous system. IL-6 is involved in the etiopathogenesis of acute or chronic neuroinflammatory diseases such as Alzheimer’s disease and Parkinson’s disease (Rojo et al., 2008). However, IL-6 is not only involved in inflammation and infection but is also related to the regulation of metabolic, regenerative, and neuronal processes. The regenerative and anti-inflammatory activities of IL-6 are mediated by gp130-associated classic signaling (Scheller et al., 2011). IL-8, known as neutrophil-activating peptide 1, is generated by monocyte-derived macrophages, microglia, and astrocytes. It functions as a trophic factor in the maintenance of normal neurons and promotes neuronal survival and angiogenesis (Langford et al., 2002). IL-9 may exert both aggravating and suppressive roles in experimental encephalomyelitis. In an experimental model, treatment with anti-IL-9 neutralizing antibodies can attenuate autoimmune encephalomyelitis (Zhou et al., 2011).

In the present study, the yield of apheresis (TNC and CD34+ cell number) on the 5th day of G-CSF administration was significantly lower in cerebral palsy children than in healthy adults, even with higher viability. We also observed that the intracellular expression of inflammatory cytokines rather than neurotrophic factors could be altered by G-CSF mobilization, when comparing the expression of PBMCs and mPBMCs between cerebral palsy children and healthy adults. IL-6 levels were commonly increased, along with the decrement of IL-3 in cerebral palsy children and the increment of IL-1β in healthy adults. Comparisons of cytokine expression between stem cell sources revealed that the expression of BDNF in mPBMCs from cerebral palsy children were significantly higher than that from the CB or mPBMCs of healthy adults. The expression of G-CSF in mPBMCs from cerebral palsy children was comparable to that from the CB, and both were significantly higher than that in healthy adults. The lower expression of pro-inflammatory cytokines (IL-1β, IL-3, IL-6) and higher expression of anti-inflammatory or trophic cytokines (IL-8, IL-9) in the CB or mPBMCs of cerebral palsy children as compared to healthy adults suggests a positive effect on neuronal regeneration, although some cytokines have both deleterious and beneficial effects on neuronal cells. These findings also suggest that the CB or autologous mPBMCs may be a potential source of cellular therapy for cerebral palsy children. In conclusion, mPBMCs from cerebral palsy children and MNCs from the CB provide a new potential source for cellular therapy for cerebral palsy children. Further investigations, including clinical trials to reveal clinical efficacy as well as therapeutic mechanisms, are warranted.

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