Infusion of Human Umbilical Cord Blood Cells Ameliorates Hind Limb Dysfunction in Experimental Spinal Cord Injury through Anti-inflammatory, Vasculogenic and Neurotrophic Mechanisms

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1. Introduction

Although enormous progress has been made in the field of cell transplantation in the last two decades, American governmental restrictions on embryonic stem cells, negative outcomes reported from clinical trials, and ethical concerns over the use of cells from fetal or embryonic sources have slowed progress in cell replacement and repair. However, we believe the most promising cells are those of hematopoietic...
origin, specifically human umbilical cord blood cells (HUCBCs). These cells have been used extensively over the last 20 years to treat various nonmalignant and malignant hematopoietic diseases, particularly in children.1,2

HUCBCs are also strong candidates for use in cell therapy for spinal cord injury (SCI) because of their availability, weak immunogenicity, and low risk of mediating viral transmission.3 For example, intraspinal transplantation of CD34+ cells derived from HUCBCs after spinal cord hemisection4 or contusion5 injury improved hind limb functional recovery in adult rats, and Saporta et al6 also reported that intravenous (IV) infusion of unfractionated HUCBCs improved hind limb function in a rat model of spinal cord compression.

Trauma to the central nervous system triggers intraparenchymal inflammation and activation of systemic immunity, with the capacity to exacerbate neuropathology and stimulate mechanisms of tissue repair.7 Despite our incomplete understanding of the mechanisms that control these divergent functions, immune-based therapies are becoming a therapeutic focus. Furthermore, cord blood lymphocytes express cytokine receptors (interleukin [IL]-2, IL-4, IL-6, IL-7, tumor necrosis factor [TNF]-α, and interferon-γ) at lower levels than adult blood cells8 and produce large amounts of the anti-inflammatory cytokine, IL-10.9 However, it remains to be determined what factors or mediators secreted from HUCBCs are crucial for restoration of the injured spinal cord in the acute stage of SCI.

2. Methods

2.1. Animals

Adult male Sprague-Dawley rats (weight, 278±14g) were obtained from the Animal Resource Center of the National Science Council of the Republic of China (Taipei, Taiwan). The animals were housed, four in a group, at an ambient temperature of 22±1°C, with a 12-hour light–dark cycle. Pellet rat chow and tap water were available ad libitum. All protocols were approved by the Animal Ethics Committee of the Chi Mei Medical Center (Tainan, Taiwan) in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and the Guidelines of the Animal Welfare Act.

2.2. Surgery

Laminectomy, with removal of the vertebral peduncle, was performed at T8/T9 on rats anesthetized with sodium pentobarbital (25mg/kg, intraperitoneal; Sigma Chemical Co., St. Louis, MO, USA) and a mixture containing ketamine (44mg/kg, intramuscular; Nankuang Pharmaceutical, Taiwan), atropine (0.02633mg/kg, intramuscular; Sintong Chemical Industrial Co., Taiwan), and xylazine (6.77mg/kg, intramuscular; Bayer, Germany). The jaws of a calibrated aneurysm clip with a closing pressure of 55g were placed between the dorsal and ventral surfaces of the spinal cord and left in place for 1 minute.10 Sham-operated control animals received the same laminectomy, but the spinal cord was not compressed. All animals were given 0.1mL of fluoroquinolone (Baytril, Bayer, Germany) antibiotic for 3 days following surgery. Animals with SCI were individually housed on special bedding to prevent skin breakdown, and had their bowels and bladders manually expressed twice daily. Food and water were freely accessible at a lowered height in their cages.

2.3. Preparation of PBMCs and HUCBCs

With approval for this project from the Institutional Review Board of Chi Mei Medical Center (Tainan, Taiwan), human peripheral blood mononucleocytes (PBMCs) and HUCBCs were obtained from freshly collected buffy coat fractions from healthy donors at Chi Mei Medical Center. HUCBCs were isolated by centrifugation over a Ficoll-Paque (Farmacia, Uppsala, Sweden) density gradient at 400g for 30 minutes at room temperature in a Sorvall 6000B (Du Pont, DE, USA) antibiotic for 3 days following surgery. The cells collected at the interface were washed three times with serum-free Roswell Park Memorial Institute (RPMI)-1640 (Gibco-BRL, Grand Island, NY, USA) and subsequently resuspended in serum-free lymphocyte medium (Gibco-BRL) and stored at 37°C in an incubator. The PBMCs at a concentration of 5×10⁶ cells in 0.3mL were prepared and stored at 37°C in an incubator. For IV administration, a 26-gauge needle was inserted into the tail vein, and cells (0.3mL) were delivered over a 1-minute period.

2.4. Experimental groups

Animals were assigned randomly to one of the following three major groups: (1) one group of rats was treated with laminectomy at T8/T9, followed immediately by IV infusion of PBMCs (5×10⁶ in 0.3mL) per rat; (2) the second group of animals was treated with laminectomy at T8/T9, followed immediately by an IV infusion of HUCBCs (5×10⁶ in 0.3mL); and (3) the third group of animals was used as a sham-operated control group.

In experiment 1, an IV dose of HUCBCs, PBMCs (5×10⁶ in 0.3mL) or saline was randomly administered immediately after SCI (n=12), and their effects on the maximal angle that animals could cling to on an inclined plane were assessed 1–7 days after SCI.
In experiment 2, an IV dose of HUCBCs, PBMCs (5×10^6 in 0.3 mL) or saline was randomly administered immediately after SCI (n=12), and their effects on the serum levels of IL-10 and TNF-α were assessed 1–7 days after SCI.

In experiment 3, an IV dose of HUCBCs (5×10^6 in 0.3 mL) or PBMCs (5×10^6 in 0.3 mL) was randomly administered immediately after SCI, and their effects on the amounts of vascular endothelial growth factor (VEGF)- and glial cell line-derived neurotrophic factor (GDNF)-positive cells in the injured spinal cord were assessed 7 days after SCI (n=12).

2.5. Inclined plane

The inclined plane was used to measure limb strength. Animals were placed, facing right and then left, perpendicular to the slope of a 20×20 cm ribbed surface on an inclined plane, starting at an angle of 55°. The angle was increased or decreased in 5° increments to determine the maximal angle at which an animal could hold to the plane. Data for each day were the mean of the left- and right-side maximal angles. All behavioral tests were independently scored by two observers who were unaware of prior treatment. These scores were averaged to arrive at a mean score for each animal for the behavioral session.

2.6. Assay of cytokines

For determination of TNF-α and IL-10, blood samples were taken 3 days after SCI, or the equivalent time for the sham-operated rats. The blood samples were allowed to clot for 2 hours at room temperature and then centrifuged (2000g, 20 minutes, 4°C). The supernatants were collected and stored at −70°C until time of measurement. The concentrations of TNF-α and IL-10 in serum were determined using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Optical densities were read on a plate reader set at 450 nm for TNF-α and IL-10. The concentrations of TNF-α and IL-10 in the samples were calculated from the standard curve multiplied by the dilution factor, and were expressed as pg/mL.

2.7. Immunohistochemistry

Autofluorescence was quenched using the method of Vendrame et al, after which the spinal cord sections were incubated with mouse monoclonal antibody against human nuclei (HuNu; Chemicon Inc., Pittsburgh, PA, USA), followed by fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (Annexin Alexa; Molecular Probes, Eugene, OR, USA). 4',6-Diamidino-2-phenylindole (DAPI) staining (Molecular Probes, Eugene, OR, USA) was performed to visualize nucleated cells. Slides were examined under epifluorescence on an Olympus BX60 microscope. For determination of VEGF and GDNF expression at the level of T8 to T9 sections, the sections were incubated with phosphate-buffered saline containing anti-VEGF and anti-GDNF mouse antibody at 1:200 dilution and then detected with Alexa-Fluor 568 (Molecular Probes, Eugene, OR, USA) goat antimouse (IgG) antibody.

2.8. Statistical analysis

Data are presented as the mean±standard error of the mean. Repeated measures analysis of variance was conducted to test the treatment-by-time interactions and the effect of treatment over time on each score. The Duncan’s multiple-range test was used for post hoc multiple comparisons among means. A p < 0.05 was considered to be statistically significant.

3. Results

3.1. HUCBCs improved motor performance after SCI

Behavioral tests of motor function were conducted on days 1, 4 and 7 after SCI to determine whether HUCBC therapy, applied immediately after SCI, could produce immediate and significant effects. As shown in Figure 1, although PBMC therapy was ineffective in all tests at these time points, the SCI-induced motor deficits, measured by the maximal angle at which an animal could hold to the inclined plane, were completely abrogated at days 4–7 after SCI by an IV dose of HUCBCs administered immediately after SCI (Figure 1).

3.2. HUCBCs decreased TNF-α but stimulated IL-10 production in serum after SCI

The serum levels of both TNF-α and IL-10 were measured on day 3 after SCI. ELISA revealed that HUCBC therapy significantly attenuated the overproduction of TNF-α (Figure 2) by 3 days after SCI. However, the serum levels of IL-10 were significantly elevated by HUCBC therapy, at 3 days after SCI (Figure 2).

3.3. HUCBCs increased both endogenous VEGF and GDNF

To demonstrate if GDNF or VEGF could be secreted by the HUCBCs in the spinal cord-injured area, sections of injured spinal cord were analyzed for VEGF and GDNF using immunofluorescence. This
staining revealed that, after 7 days, the spinal cord-injured rats treated with HUCBC infusion, but not PBMC therapy, displayed an increased number of VEGF- (∼60) and GDNF-positive cells (∼90) in their injured spinal cord sections (Figures 3 and 4), compared with those of PBMC-treated groups.

3.4. Delivered HUCBCs were localized to spinal cord injured area as determined by immunohistochemistry

Human nuclei immunoreactive cells were detected in the spinal cord-injured area of animals injected with 5 × 10^6 HUCBCs, but not with PBMCs, immediately after SCI, by DAPI staining (Figure 5). This revealed that HUCBCs could be detected in the spinal cord-injured section 7 days after transplantation.

4. Discussion

In this study, systemic infusion of HUCBCs improved functional recovery in rats with spinal cord injuries. HUCBCs are rich in hematopoietic stem cells (CD34+ cells).13 Two percent of the HUCBCs are stem cells capable of reconstituting blood lineages. These HUCBCs have been used to reconstitute bone marrow and blood cell lineages in children with malignant and nonmalignant diseases.14 In the present study, HUCBCs were transplanted via the tail vein immediately after SCI, and CD34+ cells transplanted directly into the spinal cord 1 week after injury have also been shown to improve functional recovery in rats.5 These investigators reported that transplanted CD34+ cells survived in the host spinal cord for at least 3 weeks after transplantation, but had disappeared by 5 weeks.5 In addition, it was found that IV infusion of HUCBCs alone showed therapeutic effects when administered 24 hours to 7 days after stroke15,16 or traumatic brain injury.17 The results reported here are consistent with several previous findings.4,5 In our present study, the rats were receiving no immunosuppression, which is generally a requisite for long-term graft survival but is often accompanied by deleterious side effects. However, transplantation was still able to provide neuroprotection, even under these conditions.

Davies et al18 reported that elevated serum levels of circulating proinflammatory cytokines (e.g., IL-1β, IL-6 and TNF-α) and autoantibodies were present in SCI subjects without medical complications. Hence, degradation of serum proinflammatory cytokines might be crucial for functional recovery after SCI in the acute stage. Our previous results showed that CD34+ cells exerted anti-inflammatory...
HUCBCs ameliorate SCI in rats

Effects in animal models of heatstroke. The present findings further demonstrated that HUCBC therapy caused a significant increase in the serum levels of IL-10, accompanied by a reduction in TNF-α overproduction during SCI. In fact, IL-10 possesses important anti-inflammatory properties through suppression of TNF-α and other pro-inflammatory cytokines. Our data imply that HUCBCs may improve hind limb function after SCI by increasing IL-10 and decreasing TNF-α production.

Figure 3 Vascular endothelial growth factor (VEGF)-positive cells. *The numbers of VEGF-positive cells in injured spinal cord section of rats 3 days following spinal cord injury (SCI) were significantly increased (p<0.05; n=12) for SCI animals treated with human umbilical cord blood cells (HUCBCs; 5×10⁶/0.3 mL) compared with peripheral blood mononucleocytes (PBMC)-treated groups. Representative sample of VEGF-positive cell stained spinal cord sections from animals sacrificed at 72 hours post-SCI are depicted in the upper panel.

Figure 4 Glial cell line-derived neurotrophic factor (GDNF)-positive cells. *The numbers of GDNF-positive cells in injured spinal cord of rats 3 days following spinal cord injury (SCI) were significantly increased (p<0.05; n=12) for SCI animals treated with human umbilical cord blood cells (HUCBCs; 5×10⁶/0.3 mL) compared with peripheral blood mononucleocytes (PBMCs; 5×10⁶/0.3 mL). Representative sample of GDNF-positive cell stained spinal cord sections from animals sacrificed at 72 hours post-SCI are depicted in the upper panel.
Therapeutic vascular growth is a new concept in the treatment of ischemic vascular diseases. The members of the VEGF family regulate all types of vascular growth. Various strategies have been presented for the use of post-ischemia vasculogenesis. Indeed, as shown by the present results, systemic administration of HUCBCs stimulated production of both VEGF and GDNF in the injured spinal cord. These findings support the hypothesis that HUCBCs may promote an environment conducive to revascularization of ischemic spinal cord, so that neuronal regeneration can proceed. This hypothesis is supported by several studies. A rich vascular environment, along with generation of VEGF may enhance subsequent neuronal regeneration. Summarizing these observations, it appears that HUCBCs may improve functional recovery after SCI by enhancing neovessel formation and accelerating endogenous neurogenesis.

Some researchers have reported that HUCBC grafts, instead of the host tissues per se, were likely to be the source of neuroprotective trophic factors. The expression of several neurotrophic factors in the brain are influenced by cerebral ischemia. GDNF is a well-known neurotrophic factor that promotes the survival and morphologic differentiation of dopaminergic neurons and motoneurons. Topical application of GDNF and adenovirus-mediated GDNF gene transfer significantly attenuated infarct size in a rat middle cerebral artery occlusion model, while mesenchymal stem cells that produced GDNF also lessened ischemic damage in the rat middle cerebral artery occlusion model. In our previous studies, we demonstrated that systemic exogenous administration of GDNF improved functional recovery in SCI rats. In the present study, systemic infusion of HUCBCs, but not PBMCs, significantly increased the production of GDNF in the injured spinal cord 4–7 days after injury. Thus, it is likely that HUCBCs may restore hind limb motor function in the rat SCI model by producing GDNF and/or other neurotrophic factors.

Although this study used xenografted cells, the HUCBCs did not cause critical morbidity after transplantation. This may have been because of weak immunogenicity and the short observation period of the study, as we focused on treatment of the acute stage. Further studies should aim to examine the effects of HUCBCs during chronic SCI and to compare the differences between acute and chronic SCI.
In summary, the current findings demonstrate that systemic administration of HUCBCs promotes the recovery of hind limb function in an SCI model, through stimulating the production of IL-10, GDNF and VEGF. Collection of HUCBCs by pediatricians or obstetricians in the delivery room is relatively straightforward; the procedures are easy and pose no risk to mother or baby. In addition, HUCBCs are safe to use and associated with few ethical issues, as compared with bone marrow transplantation. Thus, it appears that HUCBC therapy is a potentially useful strategy for the treatment of SCI.

Acknowledgments

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References