Original Article
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Expression of neurotrophic factors in injured spinal cord after transplantation of human-umbilical cord blood stem cells in rats

Hyo-jin Chung[1], Wook-hun Chung[1], Jae-Hoon Lee[2], Dai-Jung Chung[1], Wo-Jong Yang[1], A-Jin Lee[1], Chi-Bong Choi[1], Hwa-Seok Chang[1], Dae-Hyun Kim[1], Hyun Jung Suh[1], Dong-Hun Lee[5], Soo-Han Hwang[3,†], Sun Hee Do[4], Hwi-Yool Kim[1,*]

Departments of [1]Veterinary Surgery, [4]Veterinary Clinical Pathology, and [5]Avian Disease Laboratory, College of Veterinary Medicine, Konkuk University, Seoul 05029, Korea
[2]Department of Veterinary Surgery, College of Veterinary Medicine, Gyeongsang National University, Jinju 52828, Korea
[3]Seoul Cord Blood Bank, Histostem Co, Seoul 05372, Korea

We induced percutaneous spinal cord injuries (SCI) using a balloon catheter in 45 rats and transplanted human umbilical cord blood derived mesenchymal stem cells (hUCB-MSCs) at the injury site. Locomotor function was significantly improved in hUCB-MSCs transplanted groups. Quantitative ELISA of extract from entire injured spinal cord showed increased expression of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3). Our results show that treatment of SCI with hUCB-MSCs can improve locomotor functions, and suggest that increased levels of BDNF, NGF and NT-3 in the injured spinal cord were the main therapeutic effect.

Keywords: brain-derived neurotrophic factor, nerve growth factor, neurotrophic factor, neurotrophin-3, spinal cord injury

Introduction

Traumatic spinal cord injuries (SCI), including compression and contusion, may show remaining axons in the periphery of the lesion, but they are non-functional due to demyelination. Demyelination of axons is the result of the primary mechanical trauma, followed by secondary damage, which may include edema, ischemia, inflammation and excitotoxicity [3,18,32]. Although some spontaneous remyelination occurs, spontaneous regeneration of demyelinated axons is limited, and research regarding SCI focused primarily on promotion of axonal regrowth, remyelination and reduction of degenerative change [3,6,10,23,31]. Cell-based therapy has been considered to promote remyelination and functional recovery of SCI. Recent studies have included the use of transplanted oligodendrocyte precursor cells [15,16], bone marrow stromal cells [35], embryonic stem cells [24] and olfactory glial cells [13].

Human umbilical cord blood derived mesenchymal stem cells (hUCB-MSCs) are one of the attractive sources for cell transplantation in SCI. hUCB-MSCs are less mature than bone marrow stems cells, and therefore less immunogenic [7]. Furthermore, hUCB-MSCs showed more proliferation potential associated with extended life span [7], raised less ethical problems and could be cryopreserved without a significant decline in viability [9].

In previous studies, hUCB-MSCs could be differentiated into various neural cells and showed improvement of locomotor function [5,19,20]. hUCB-MSCs secreted various cytokines that could be beneficial to SCI recovery, both *in vitro* and *in vivo* [3,6,9]. Moreover, Lee *et al*. [21] recently reported that hUCB-MSCs transplanted into dogs with SCI were able to facilitate Schwann cell-like myelination. Many studies have shown that tissue regeneration elicited by transplanted mesenchymal stem cells (MSCs) may not be the main source of functional recovery, since only a small proportion of MSCs appeared to differentiate into glial cells. Thus, increased production of neurotrophic factor following transplanted MSCs may be among the possible mechanisms of MSC-induced functional recovery [18,24,28].

In this study, we transplanted hUCB-derived MSCs into the

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*Corresponding author: Tel: +82-2-450-3664; Fax: +82-2-446-9876; E-mail: hykim@konkuk.ac.kr
[†]Present address: Stem Cell Technology Center, CELNESS Co., Anyang 14048, Korea
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injured spinal cord to evaluate functional recovery, and demonstrated increased expression of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) by transplanted hUCB-MSCs.

Materials and Methods

Spinal cord injury

All experimental protocols were conducted according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University, Seoul, Korea (KU09072). Forty-five male, 9-week-old, 290 to 330 g Sprague-Dawley rats were used and divided into two groups as described in Table 1.

Anesthesia was induced using a 3% isoflurane chamber (Forane; JW Pharmaceutical, Korea), and maintained by 2 to 2.5% isoflurane. An 18G spinal needle (Weiss, Fixed Wing, Modified Tohy Point; Becton, Dickinson and Company, USA) was placed into the epidural space via the lumbosacral joint under fluoroscopic guidance (Mobile C-RAM System; MCA-6100; Medison Xray, Korea), and a 2Fr Fogarty catheter was inserted into the epidural space through the spinal needle under fluoroscopic guidance. The 2Fr Fogarty catheter was filled with half-strength iohexol (Omnioaque; Amersham Health, Ireland) and connected to a 50 μL Hamilton syringe (type 1705). The tip of the balloon catheter was placed at T9 and inflated to 50 μL for 10 min using half strength iohexol. After confirming position and shape by fluoroscopy, the balloon catheter was removed following deflation. No antibiotics were given post-procedure. Manual bladder expression was performed twice daily [5].

Harvest and preparation of hUCB-derived MSCs

hUCB-derived MSCs were prepared as previously described [11], with some modifications. Shortly, through the donor who has agreed with written, informed consent, human umbilical cord blood (UCB) samples were freshly obtained from full-term deliveries. By using a Ficoll-Paque Plus kit (GE Healthcare, Sweden), mononuclear cells (MNCs) were isolated from the low-density mononuclear fraction (MNC $<1,077$ g/mL). Total MNCs were grown in DMEM low glucose culture medium (Gibco-BRL, USA) which contains 20% fetal bovine serum (FBS; Gibco-BRL), including basal fibroblast growth factor (bFGF; 10 ng/mL), stem cell factor (SCF; 10 ng/mL), 100 U penicillin, 1,000 U streptomycin, and 2 mM L-glutamine (Gibco-BRL). Grown total MNCs were then plated in T-25 flasks at a concentration of 5×10^6 cells/cm^2. UCB cells were maintained at 37°C in an incubator containing 5% CO_2 under a humidified atmosphere. Culture medium was replaced every 3 days. From attached cells, MSCs were passaged by trypsinization (0.005% trypsin/EDTA; Gibco-BRL). Confluence was reached upon 80 to 90% at 5×10^4 cells/cm^2 in T-25 flasks. Spindle-shaped homogeneous MSCs populations were trypsinized at the second or third passage. Characterization and differentiation of isolated hUCB-derived MSCs were performed as previously described using the same cell source and isolation technique [4,11,14]. The hUCB-derived MSCs were provided for pure research purposes by the Seoul Cord Bank (Histostem, Korea).

Stem cell transplantation

Transplantation of hUCB-derived MSCs was performed at 3 days after SCI under general anaesthesia. An incision was made in the skin and the muscle was separated (from the near side of the spinous process at T9−T10 and confirmed spinous process), after which 10 μL of saline was administered to the CytoCon group in three spinal cord segments, cranial (T8−T9) and caudal (T10−T11) to the injury site, and directly to the injured site (T9−T10). The CytohUCB group was administered a total of 2×10^5/30 μL of hUCB-MSCs, which was divided into three parts and each 10 μL of hUCB-MSCs was administered in three spinal cord segments. Immunosuppressants were not administered in these cases.

Enzyme linked immunosorbent assay (ELISA)

Five animals in the CytoCon group were sacrificed at 3 days after SCI, then five animals each from the CytohUCB group and CytoCon group were sacrificed at 7, 14, 21 and 28 days after SCI. Following sacrifice, the spinal cord (T7−T12) was extracted and homogenized with a cocktail of proteases inhibitors (Sigma, USA) in RIPA buffer (Millipore, USA), then centrifuged at 10,000 × g and 4°C. Supernatants were harvested and quantitative analysis of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3

Table 1. Experimental design and description of groups

Group number	Group description	Transplantation category	Transplantation segment	Transplantation day	Transplantation dose	Analysis	Number of animals
1	CytohUCB	hUCB	3 segment	3 days after SCI	2×10^5/30 μL	ELISA	20
2	CytoCon	Saline	3 segment	3 days after SCI	30 μL of saline	ELISA	25

hUCB, human umbilical cord blood; SCI, spinal cord injury; ELISA, enzyme-linked immunosorbent assay.

(NT-3) was conducted using an ELISA kit (Boster Biological Technology, China).

Statistical analysis

Statistical analyses were performed using SPSS (ver. 17.0; SPSS, USA). All data were analyzed by a Student's *t*-test or Mann-Whitney *U* test. *P* values < 0.05 were considered statistically significant.

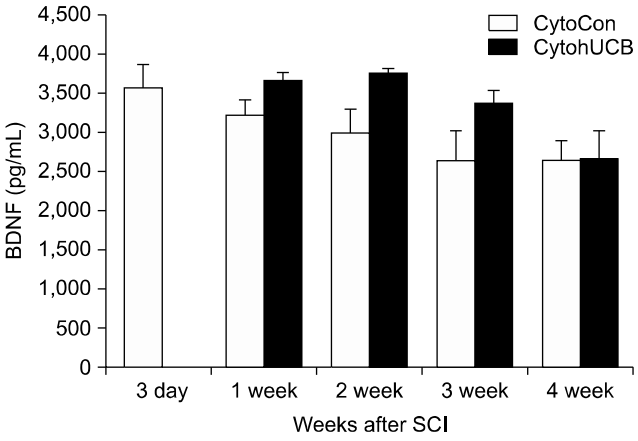

Fig. 1. Expression of brain-derived neurotrophic factor (BDNF) in the injured and treated spinal cord in rats. BDNF expression level decreased consistently from 3 days to 4 weeks after SCI in the CytoCon group. However, expression was maintained at the same level until week 3 after SCI in the CytohUCB group. Results are presented as the mean ± SEM ($p < 0.05$).

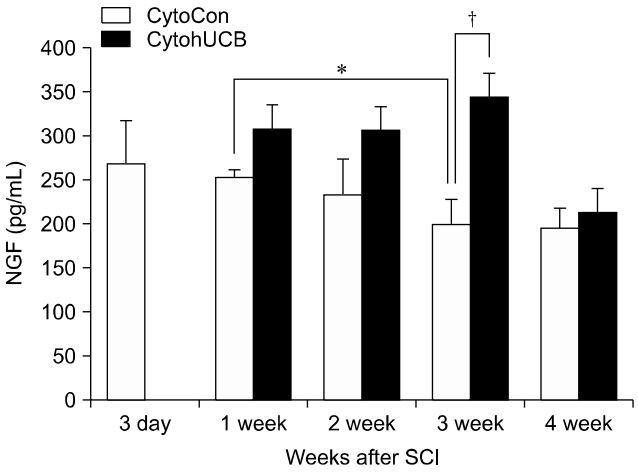

Fig. 2. Expression of nerve growth factor (NGF) in injured and treated spinal cord in the rat. The NGF was consistently decreased from 3 days to 4 weeks after SCI in the CytoCon group. In contrast, the CytohUCB group showed a consistent increase from 3 days to 3 weeks after SCI. At 3 weeks after SCI, there were significant differences between the CytohUCB group and CytoCon group ($p < 0.05$). The results are presented as the mean ± SEM.*$p < 0.05$, †$p < 0.05$.

Results

Tissue levels of BDNF, NGF and NT-3

In this study, we performed quantitative ELISA analysis by extracting BDNF, NGF and NT-3 from spinal cord tissue. hUCB-MSCs were transplanted 3 days after SCI and measurements were conducted 3 days, 1 week, 2 weeks, 3 weeks, and 4 weeks after SCI. In the CytoCon group, BDNF decreased consistently from 3 days to 4 weeks after SCI. However, in the CytohUCB group transplanted with hUCB-MSCs, BDNF level remained the same until 3 weeks after SCI. In the CytohUCB group, BDNF decreased 4 weeks after SCI (Fig. 1). In the CytoCon group, NGF consistently decreased from 3 days to 4 weeks after SCI. In contrast, the CytohUCB group showed a consistent increase in NGF from 3 days to 3 weeks after SCI. On week 3, there was a significant difference between the CytohUCB group and the CytoCon group ($p < 0.05$). Similarly, NGF decreased 4 weeks after SCI, as in the CytoCon group (Fig. 2). In addition, NT-3 was increased in both the CytohUCB group and the CytoCon group at 3 days after SCI, but that in the CytoCon group at 2 weeks after SCI decreased until 4 weeks after SCI. However, the hUCB-MSCs transplanted CytohUCB group showed a consistently increased level from 1 week to 4 weeks after SCI, and there was a significant difference between the CytohUCB group and the CytoCon group at 2 weeks after SCI ($p < 0.05$) (Fig. 3).

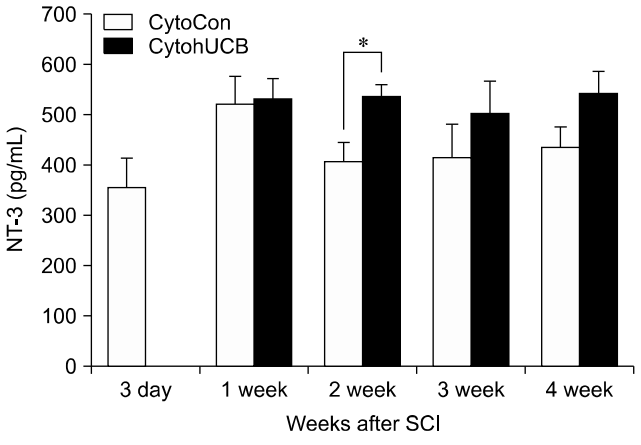

Fig. 3. Expression of neurotrophin-3 (NT-3) in injured and treated spinal cord in the rat. The NT-3 expression in both CytohUCB and CytoCon groups increased 1 week after SCI relative to SCI 3 days after SCI, but in the CytoCon group the level decreased from 2 weeks after SCI until 4 weeks after SCI. However, the CytohUCB group showed consistent levels from 1 week to 4 weeks after SCI, and there was a significant difference between the CytohUCB group and CytoCon group at 2 weeks after SCI ($p < 0.05$). The results are presented as the mean ± SEM. *$p < 0.05$.

Discussion

In cell therapy, hUCB cells have numerous advantages over other sources of stem cells. Specifically, hUCB-MSCs are more pluripotent and genetically more flexible than bone marrow mesenchymal stem cells. Furthermore, they are less mature and therefore less immunogenic than other adult stem cells [7,8,11]. hUCB-MSCs are considered to have the therapeutic potential to replace damaged tissue by proliferation and differentiation. Alternatively, they may repair damaged tissue and protect neurons by secretion of neurotrophic factor [29].

Both *in vitro* and *in vivo* hUCB-MSC have been reported to differentiate into astrocytes, neurons and oligodendrocytes [4,6,20]. However, according to Chua *et al*. [3], transplanted hUCB-MSCs were found until 1 week after transplant, but were not found at 3 weeks after transplantation. In addition, after hUCB-MSCs transplantation, Ha *et al*. [12] reported that only 2% of cells were positive for the astrocyte marker GFAP and that a similar percentage or less were positive to the neurofilament marker MAP-2. These findings suggested that, *in vivo*, hUCB-MSCs may differentiate into either neuron or glial cells, but that the frequency is low and therefore unlikely to create a therapeutic effect. In contrast, the neurotrophic factor, growth factor and cytokines secreted from hUCB-MSCs may lead to remyelination of demyelinated axons and express therapeutic effects [3].

Many studies have reported that neurotrophic factors play an important role in spinal cord regeneration in SCI. Several studies have reported neuroprotective effects and axonal regeneration promotion with NT-3 after SCI in adult rats [2,17,26,30,31]. Shang *et al*. [33] transplanted NT-3 secreting human umbilical mesenchymal stem cells (NT-3-HUMSCs) in injured spinal cord and reported that NT-3-HUMSCs could promote the morphological and functional recovery. Moreover, administration of NT-3 antibody after spinal cord transection in rats led to decreased endogenous NT-3 expression and corticospinal tract regeneration compared with the control group and somatosensory evoked potentials could not be detected. Therefore, NT-3 could play an important role in spinal plasticity in injured spinal cord [36]. BDNF prevented atrophy of rubrospinal neurons and reduced the extent of the lesion cavity after spinal cord injury in rats [34]. Kuh *et al*. [19] compared the therapeutic effects of hUCB and hUCB with BDNF in SCI rats. The hUCB with BDNF transplantation group showed a greater improvement in locomotor function than the hUCB transplantation group. NGF has also been shown to promote axonal regeneration and preserve neural tissue after spinal cord injury in rats [18]; therefore, we measured BDNF, NGF and NT-3 in injured spinal cord.

In numerous studies, secretion of neurotrophic factor was demonstrated by immunostaining and RT-PCR analysis, both *in vitro* and *in vivo* [1,3,4,6,9]. In this study, neurotrophic factors such as BDNF, NGF and NT-3 were transplanted with hUCB-MSCs and analyzed by quantitative ELISA in tissue homogenate.

Qin *et al*. [27] examined the levels of endogenous BDNF, NGF and NT-3 after spinal cord hemisection in the rat and reported increased levels 3 days after SCI. Specifically, NGF levels increased at 7 days after SCI, whereas BDNF and NT-3 levels peaked at 3 days after SCI, then consistently decreased. In another study, the level of NT-3 peaked 7 days after SCI [36], while Li *et al*. [22] suggested that NGF and BDNF peaked 7 days and NT-3 peaked 2 weeks after spinal cord transection in the rat [22]. In most cases, endogenous neurotrophic factor has been reported to peak between 3 days and 7 days after spinal cord lesion. In this study as well in the control group, NGF and BDNF peaked at 3 days after SCI, whereas NT-3 peaked 1 week after SCI. However, NGF and BDNF were found to be maintained until 3 weeks after SCI and NT-3 until 4 weeks after SCI, respectively, without showing a decrease in the hUCB-MSCs group. These results suggested that hUCB-MSCs function to secrete BDNF, NGF and NT-3 within the injured segments of the spinal cord. Neuroprotection and axonal regeneration elicited by BDNF, NGF and NT-3 may also be promoted for more than 2 weeks after SCI compared to control group.

In the present study, there were significant increases in BDNF, NGF and NT-3 after hUCB-MSCs transplantation was confirmed. BDNF and NGF were decreased 3 weeks after SCI, and NT-3 was maintained until 4 weeks after SCI; therefore, it is assumed that additional transplantation of hUCB-MSCs at 3 weeks after SCI may induce more protective effects compared with single transplantation.

Overall, this study revealed that the therapeutic potency of transplanted hUCB-MSCs occurs by increasing the levels of BDNF, NGF and NT-3 in the injured spinal cord.

Acknowledgments

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Conflict of Interest

There is no conflict of interest.

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