Neurally induced umbilical cord blood cells modestly repair injured spinal cords

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Received 3 May 2008; accepted 11 May 2008

DOI: 10.1097/WNR.0b013e3283089234

Umbilical cord blood (UCB) is known to have stem/progenitor cells. We earlier showed that novel progenitors could be isolated from cryopreserved human UCB with high efficiency. The multipotent progenitor cells were induced to differentiate into neural-lineage cells under the appropriate condition. In this study, we confirmed these neurally induced progenitor cells (NPCs), containing higher quantities of nerve growth factor, promoted functional recovery in rats with spinal cord injury (SCI). Sprague–Dawley rats with SCI achieved a modest improvement in locomotor rating scale until 10 weeks after transplantation of the NPCs. SCI rats treated with NPCs also showed somatosensory-evoked potentials were recovered, and grafted cells especially exhibited oligodendrocytic phenotype around the necrotic cavity. These findings suggest that UCB-NPCs might be a therapeutic resource to repair damaged spinal cords. *NeuroReport* 19:1259–1263 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Keywords: functional recovery, multipotent progenitor cells, neural induction, spinal cord injury, umbilical cord blood

Introduction

Transplantation using stem cells or progenitors from adult tissue such as bone marrow (BM) [1–3] and umbilical cord blood (UCB) [4–6] might provide a therapeutic approach for spinal cord injury (SCI). Hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) derived from BM or UCB have been gradually utilized as easily accessible transplantable cells, in contrast to embryonic stem cells that might yield teratomas and entail ethical issues. Recently, HSCs and MSCs have been shown to differentiate into neural-lineage cells upon induction with β-mercaptoethanol [7], retinoic acid (RA) [8], basic fibroblast growth factor [9,10], or nerve growth factor (NGF) [11].

Immature stem-like cells from human UCB seem to have a higher proliferative capacity during the early phases of the culture than those derived from adult BM [11], whereas autologous BM cells have a limitation to amplify *in vitro* over a short period to meet the needs for clinical treatment. Human UCB is also readily available via cryopreservation in anticipation of therapeutic application, and can be routinely harvested without risk to the donor [12]. Moreover, we observed earlier that multipotent progenitor cells (MPCs), which were isolated in higher yields than those of MSCs or HSCs, could be separated from cryopreserved UCB with the potential to develop into neural tissue-specific cell types under specialized medium *in vitro* [11]. This study was carried out to investigate whether the neurally induced progenitor cells (NPCs) could replace lost neural cells, remyelinate injured axons, or restore function in rats with SCI.

Materials and methods

Cell culture and neural induction

We isolated and maintained MSCs and MPCs from cryopreserved human UCB-mononuclear cells as described in earlier studies [11,12]. Thereafter, MPCs were collected at 4×10^4 cells/cm^2 and plated on poly-L-lysine-coated 96-well plates under the specific condition for neural induction. The induction media was made up of high glucose Dulbecco's modified eagle's medium, 1% fetal bovine serum, 10 μM

all-trans-RA (Sigma-Aldrich, Missouri, USA), 100 ng/ml NGF (R&D Systems, Minnesota, USA), and 10 μM forskolin (Sigma). The cells were incubated in a humidified atmosphere with 5% CO_2 for 10–14 days at 37°C.

Reverse transcriptase-PCR analysis

Total RNA was extracted using TRIzol Reagent (Gibco-BRL, Maryland, USA), and 2 μg of the RNA was reverse transcribed with AMV reverse transcriptase XL (TaKaRa, Shiga, Japan) for 90 min at 42°C. PCR was performed with Taq polymerase for 30 cycles using the following primers: Nestin, forward: 5′-GCCCTGACCACTCCAGTTTA-3′, reverse: 5′-GGAGTCCTGGATTTCCTTCC-3′; NeuroD, forward: 5′-TGACCAAATCGTACAGCGAGAG-3′, reverse: 5′-AGAAGTTGCCATTGATG CTGAGCG-3′; glial fibrillary acidic protein (GFAP), forward: 5′-ACCAGGACCTGCTC AATGTC-3′, reverse: 5′-ATCTCCACGGTCTTCACCAC-3′; myelin basic protein (MBP), forward: 5′-GTGTGAAGATC ACGTTCCTT-3′, reverse: 5′-TGAGAGAAGGACAGGAAA AA-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5′-ATCACCATCTCCAGGAGCG-3′, reverse: 5′-GT TCTTCCACCACTTCGTCC-3′. The amplified complementary DNA fragments were electrophoresed through a 1% agarose gel, stained with ethidium bromide, and photographed under an ultraviolet light transilluminator.

Immunocytochemistry

The cells were fixed with 4% paraformaldehyde in 0.1 M DPBS for 10 min, treated with 0.3% H_2O_2 in 100% EtOH for 10 min to eliminate endogenous peroxidase activity, and incubated with 10% goat or rabbit serum (Zymed, California, USA) for 1 h at 37°C. This was followed by incubation with a primary antibody specific for neurofilament-L (Chemicon, California, USA), neuron-specific enolase (SantaCruz, California, USA) or GFAP (SantaCruz) at 4°C overnight. The cells were then incubated with fluorescein isothiocyanate-conjugated or rhodamine-conjugated secondary antibodies for 1 h at 37°C, stained with 4′, 6-diamino-2-phenylindole (1 μg/ml, Sigma) to visualize nuclei.

Enzyme-linked immunosorbent assay

In the supernatants collected from the confluent layers of human UCB-derived MSCs, MPCs, and NPCs, protein levels of the neurotrophic factors such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), NGF, and NT-3 were quantified using two-site ELISA systems (Emax Immunoassay System; Promega, Wisconsin, USA). Namely, the cells of each group were maintained with optimized medium of Dulbecco's modified eagle's medium in 96-well plates for 2 days after washed with PBS three times at 10-min intervals. The media did not contain any supplements such as fetal bovine serum, RA, forskolin, and NGF to make sure this did not confound the measurement of neurotrophic factors from the supernatant. The cell supernatants were then assayed according to the manufacturer's protocols.

Animal

Sixty-one male adult Sprague–Dawley rats weighed 300–350 g at the time of surgery were used in this study. The rats were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and given food and water *ad libitum* with alternate 12-h light/dark cycles. The Institutional Review Board approved the experimental procedures.

Spinal cord injury

All rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and administrated prophylactic atropine sulfate (0.8 mg/kg, i.p.) to reduce tracheal secretions. Laminectomies were performed at the T9 level, leaving the dura mater intact. SCI were obtained by dropping a 10-g impact rod from a 25-mm height onto the exposed dorsal surface of the spinal cord using a NYU weight-drop device (New York University, New York, USA). During the recovery period, their body temperatures were maintained at 37°C in a heating chamber. Postoperative care included bladder expression one or two times per day until the animals recovered bladder function. Prophylactic kanamycin (1 mg/kg) was administered for 1 week after surgery.

Cell transplantation

After 1 week, the rats with SCI were randomly assigned to the following four groups without bias: MSCs (n=13), MPCs (n=16), NPCs (n=12), and PBS (n=20) treated groups. Rats with asymmetrically affected hindlimbs were excluded from this experiment. Using a 25-guage cannula connected to a 10 μl Hamilton syringe fixed in a stereotaxic frame (Stoelting Co., Illinois, USA), 10 μl of the cultured cells (2.5×10^5 cells) or PBS was injected into the epicenter of the injury over a 10-min period (infusion rate, 60 μl/h). All rats received cyclosporine A (10 mg/kg, i.p.) daily from 2 days before the transplantation until the completion of this experiment in order to suppress undesirable immune responses.

Behavioral assessment

A behavioral test, scored by the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale, was performed to measure motor recovery of the rats with SCI. This scale measures hindlimb movements with scores ranging from 0 (no observable movement) to 21 (normal movement), with a higher score given for the increased movement of individual joints, limb coordination, and weight-supported plantar stepping. Two independent investigators, blinded to which group the animals belonged, observed the hindlimb movements in an open field for 5 min. Locomotor functions were scored once a week from 1 day to 13 weeks after surgery. The final score was obtained by averaging those of both sides of the limbs.

Electrophysiological study

Two months after transplantation, the rats of each group (n=8) were anesthetized with urethane (1.25 gm/kg, i.p.) and pancuronium bromide (1.0 mg/kg, i.p.) to induce muscle relaxation. The rats were then intubated via tracheostomy and artificially respired using an animal respirator (Harvard Apparatus, Massachusetts, USA). The left sciatic nerve was exposed, and a pair of electrodes was attached to the nerve. A single square pulse of electrical stimulus was delivered with a 0.1-ms pulse duration and 6 mA intensity at 1–4 Hz. For somatosensory-evoked potentials (SSEPs) recording, a 4×4-mm sized craniectomy was performed in the contralateral frontoparietal area. A special recording electrode (NE-120, Rhodes Medical Instruments, California, USA) was fixed on the sensorimotor cortex at 2 mm posterior to the bregma and 2 mm lateral to sagittal suture. SSEPs consisted of 100 single sweep epochs on average. The data were analyzed from the standard representative waveforms of evoked potentials stimulated with a 6 mA intensity.

Immunohistochemistry

The rats of each group (n=5) were sacrificed and fixed transcardially with ice-cold PBS and 4% paraformaldehyde. The 20-mm transverse segments of the SCI region were dissected, stored in the same fixative overnight. The tissues were frozen and cryosectioned as 12-μm thick longitudinal sections via cryomicrotome (Microm/HM500V, Walldorf, Germany). Individual sections were immunostained for human nuclear protein (HuNu, mAb 1281, Chemicon) and one of the following markers overnight: (i) βIII-tubulin, using mAb TuJ1 (Covance, New Jersey, USA), (ii) GFAP (Chemicon), or (3) MBP (Chemicon). They were then incubated with Alexa 488 or 563 secondary antibody for 1 h, washed, and mounted on glass slides with fluorescent mounting medium (Vectorshield, Vector Laboratories, California, USA). The sections were examined under a fluorescence microscope (BX51, Olympus, Tokyo, Japan) or an argon and krypton laser scanning confocal imaging system (LSM 510, Zeiss, Gottingen, Germany). Double-stained cells were confirmed by visualizing the colocalization of HuNu and specific markers at a magnification of $\times 600$.

Results

Isolation of multipotent progenitor cells and neural induction

MPCs, homogeneous fibroblast-like cells, were harvested with more than 90% viability from cryopreserved UCB units. The cells then exposed to neural induction medium exhibited a neural-lineage cell morphology expressed by neurofilament-L, neuron-specific enolase, or GFAP, whereas the cells were not exposed to the induction medium did not express any neural-lineage cell markers (Fig. 1a–d). Semi-quantitative reverse transcriptase-PCR analysis also disclosed an increase in nestin, GFAP, and MBP by day 14, and an increase in NeuroD expression levels by day 3 which is a transcription factor transiently expressed when they start neuronal differentiation [13] (Fig. 1e and f).

Expression of neurotrophic factors *in vitro*

Whereas MSCs (n=3) and MPCs (n=18) expressed no detectable levels of the neurotrophic factors, the optimized culture supernatant from NPCs (n=19) contained significant quantities of NGF (117.05 ± 17.35 pg/ml/0.5×10^6 cells) compared with levels observed in the culture of MSCs (1.74 ± 1.01 pg/ml/0.5×10^6 cells) and MPCs (0.42 ± 0.14 pg/ml/0.5×10^6 cells). The levels of GDNF, NT-3, and BDNF were, however, not significantly different among the supernatants of MSCs (n=3), MPCs (n=10), and NPCs (n=10). When the quantities of NGF were measured at 0, 6, 12, 24, and 48 h after neural induction, human UCB-derived NPCs (n=4) significantly produce NGF in a time-dependent manner, whereas another control medium without NPCs had 5.0 pg/ml at 0 h, but did not show any detectable levels of NGF thereafter (Fig. 1g).

Behavioral testing

Before transplantation, there were no differences in the BBB scores among each group at day 1 and 7 after SCI. All of the rats seldom moved their hindlimbs 1 day after SCI but they exhibited a gradual improvement over time. One week after transplantation, NPCs-treated rats started to show an early increment over those who received PBS. Overall, the NPCs-treated group achieved a significant improvement in locomotor performance to a final score of 11.25 ± 0.48 compared with PBS group (9.0 ± 0.46) 8 weeks post-transplantation ($P < 0.05$) (Fig. 2a). Neither MSCs-treated nor MPCs-treated rats showed a significant improvement compared with PBS controls at any time, although they tended to achieve better hindlimb movement. When a part of each group, that is, MSCs (n=5), MPCs (n=4), NPCs (n=4), and PBS (n=5), continued to be evaluated for 13 weeks to see if locomotor activities were maintained for long-term period, NPCs-treated rats with SCI still exhibited a modest improvement in BBB scale to a final score of 11.25 ± 0.55 compared with PBS group (9.3 ± 0.49) ($P < 0.05$). Their hindlimb movements were, however, maintained as maximum score of 12.0 ± 0.47 until 10 weeks after transplantation of the NPCs, and they did not show any further increment thereafter as compared at each time (Fig. 2a).

Electrophysiological study

The latencies of SSEPs measured from the onset of the first negative deflection peak (N1 latencies) in rats treated with NPCs (25.93 ± 2.27 ms) were significantly shortened compared with those of the animals injected with PBS (33.88 ± 3.10 ms) ($P < 0.05$). The NPCs-treated group also

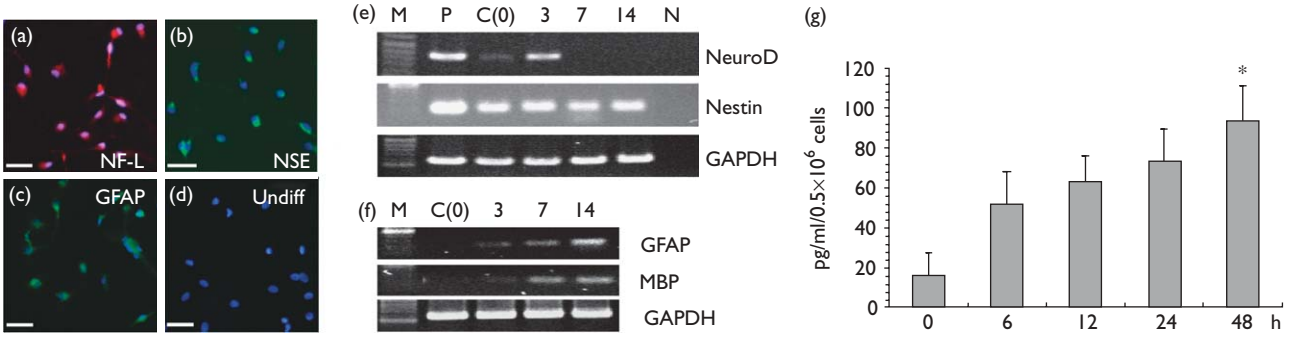

Fig. 1 Neural induction of UCB-MPCs. Neurally differentiated cells were stained for NF-L (a), NSE (b), or GFAP (c) whereas the undifferentiated cells did not express neural-lineage marker NSE (d). Nuclei were stained with 4′, 6-diamino-2-phenylindole. Scale bars: 20 μm. (e and f) reverse transcriptase-PCR was performed with primers corresponding to NeuroD, Nestin, GFAP, MBP, and GAPDH. (g) The NGF quantity of the supernatants, measured using ELISA, showed that UCB-NPCs produced NGF in a time-dependent manner. *$P < 0.05$, repeated measure ANOVA C (0), undifferentiated cells, GFAP, glial fibrillary acidic protein; M, size marker; MBP, myelin basic protein; N, negative control; NF-l, neurofilament-L; NGF, nerve growth factor; NSE, neuron-specific enolase; P, positive control (IMR32).

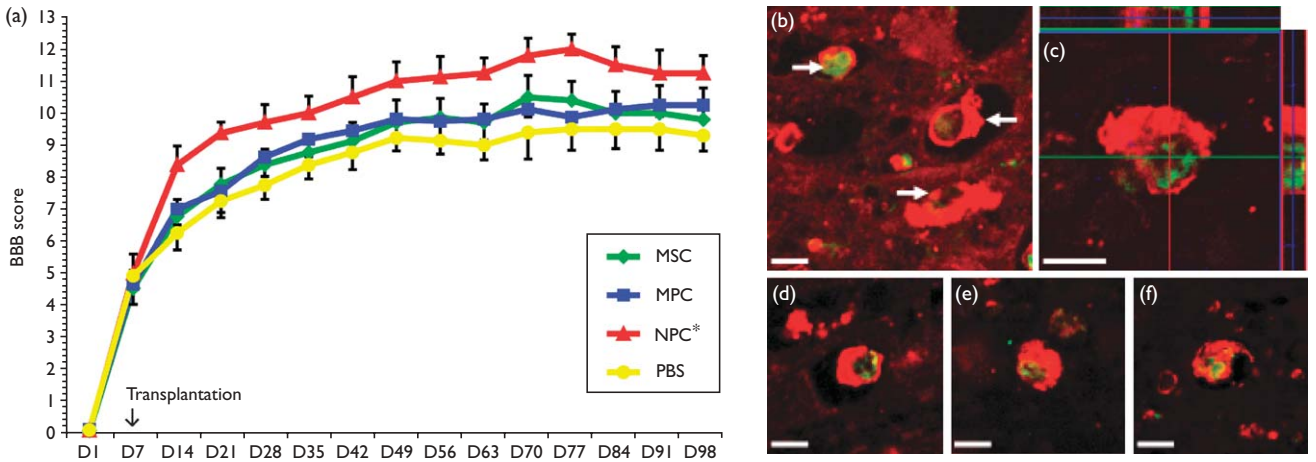

Fig. 2 Locomotor recovery and immunohistochemistry after transplantation of UCB cells. (a) Hindlimb functions were measured by BBB locomotor rating scale. NPCs-transplanted rats exhibited a significant improvement in overall locomotor performance compared to those who received PBS. Values are mean ± SE. $^{*}P < 0.05$, repeated measure ANOVA with *post hoc* comparison. (b–f) Grafted NPCs were recognized 4 weeks post-transplantation by confocal imaging of HuNu (green) colabeling with MBP (red). Arrows denote double-labeled cells. Scale bars: 10 μm. HuNu, human nucleic protein; MBP, myelin basic protein.

Table 1 Electrophysiological study in rats treated with UCB cells after posttransplantation 8 weeks

	Latency				Amplitude			
SSEP	N1 (ms)	Prolonged (times)	P1 (ms)	Prolonged (times)	Negative peak (μV)	Ratio (%)	Peak-to-peak (μV)	Ratio (%)
MSC (n=8)	28.03 ± 1.83	1.89	55.95 ± 5.23	1.65	3.40 ± 1.15	13.31	10.01 ± 3.47	14.05
MPC (n=8)	27.48 ± 1.54	1.85	53.39 ± 4.63	1.58	4.44 ± 1.54	17.38	13.74 ± 4.55	19.28
NPC (n=8)	25.93 ± 2.27†	1.74	57.30 ± 5.47	1.69	9.28 ± 2.94*,†	36.32	25.17 ± 8.30*,†	35.32
PBS (n=8)	33.88 ± 3.10	2.28	65.09 ± 5.60	1.92	3.29 ± 1.18	12.88	8.39 ± 2.85	11.77
Normal (n=5)	14.86 ± 2.27		33.86 ± 1.38		25.55 ± 6.18		71.27 ± 18.34	

Values are mean ± SE. Prolonged latency (times) and ratio of amplitude (%) are the values compared with those of intact spinal cord with laminectomy alone. $^{*}P < 0.05$ compared with MSC; $^{\dagger}P < 0.05$ compared with PBS; MPC, multipotent progenitor cell; MSC, mesenchymal stem cell; NPC, neurally induced progenitor cell; PBS, phosphated buffered saline; SSEP, somatosensory-evoked potential; UCB, umbilical cord blood.

showed a larger negative peak amplitude and peak-to-peak amplitude than rats received with MSCs and PBS ($P < 0.05$) (Table 1). When the latencies and amplitudes of SSEPs were measured in rats with intact spinal cords with laminectomy alone (n=5) to indicate how much recovery has been shown in the transplanted animals, NPCs-treated rats had 1.74 times prolonged N1 latency, 1.69 times prolonged P1 latency, 36.33% negative peak amplitude, and 35.31% peak-to-peak amplitude than those of intact spinal cord, whereas PBS-treated rats had 2.28 times prolonged N1 latency, 1.92 times prolonged P1 latency, 12.86% negative peak amplitude, and 11.77% peak-to-peak amplitude (Table 1).

Histological assessment

Double immunoreactivity for HuNu and GFAP, MBP, or βIII-tubulin confirmed that the grafted cells differentiated into neural-lineage cells and they were scattered adjacent to the epicenter of the spinal cord in MSCs, MPCs, or NPCs-treated animals 4 weeks posttransplantation. Particularly, a large percentage of HuNu-positive UCB cells in rats treated with NPCs displayed colabeled reactivity for MBP (Fig. 2b–f). When we randomly investigated 150 HuNu-positive cells from different tissue sections (n=5), we found that the percentage of HuNu-reactive cells expressing MBP was 23.86 ± 7.32% whereas those of HuNu/GFAP^{+} and HuNu/βIII-tubulin^{+} were 4.0 ± 4.0% and 1.82 ± 1.82%, respectively, in NPCs-treated rats ($P < 0.05$). Similarly, the percentage of HuNu/MBP^{+} in the NPCs group was significantly higher than that of MSCs (1.54 ± 1.54%), MPCs (9.62 ± 4.37%), or PBS (none) treated animals ($P < 0.05$), although there was no difference in the percentage of HuNu-labeled UCB cells expressing GFAP or βIII-tubulin among all of the groups. Only a few cells were, however, observed in the spinal cord of the same groups 8 weeks posttransplantation, suggesting that most of the settled cells did not survive until this time.

Discussion

Human UCB cells will be increasingly used to treat clinical diseases because they are able to be frozen and stored for future therapeutic use. We already reported that MPCs, phenotypically novel cell type, were isolated from almost all UCB harvests (95.5%) whereas MSCs were obtained at a very low frequency (<10%) [11,12]. In addition, MPCs could differentiate into neurons (60–65%), astrocytes (10–20%), and oligodendrocytes (5–10%) under in-vitro neural induction medium [11]. In this study, the NPCs were confirmed

by reverse transcriptase-PCR and immunocytochemistry, and expressed a high level of NGF as disclosed by ELISA. This study also demonstrated that intraspinal transplantation of the NPCs promoted small but significant recovery of both motor and sensory function in rats with SCI. Furthermore, grafted cells positive for HuNu also differentiated into neural tissue-specific cells that mainly expressed an oligodendrocytic marker 4 weeks after transplantation, suggesting that *in vivo* state after cellular transplantation was different from *in vitro* condition largely induced to differentiate into neurons. Electrophysiological study confirmed that the latencies of SSEPs were significantly shortened and the amplitudes of the SSEPs were increased in rats treated with NPCs, although there was some recovery in all transplanted groups compared with the PBS-injected group. This observation can be explained by the effect of the NPC-derived oligodendrocytes and consequent myelination.

Only a few of the integrated UCB cells were, however, observed at 8 weeks posttransplantation, implying a high-cell mortality rate. Nonetheless, NPC-treated animals showed improvement in the BBB locomotor rating scale compared with PBS controls by 10 weeks after transplantation. They did not maintain motor function after this time when their locomotor activities were, however, evaluated for long-term period. These results suggest that functional recovery might result from the therapeutic effect more than from the integration of NPCs, which results from neurotrophic support of the surviving or replaced cells by trophic factors secreted by grafted cells. This can be explained by reports that neurotrophic factors enhanced functional recovery by viral vector-mediated gene transfer [14], by NGF-producing cells [15], and in combination with a transplantation of UCB cells [6]. Lu *et al.* [16] also suggested that transplanted stem cells could promote extensive host axonal growth and neural repair at least in part by secreting neurotrophic factors. As NPC-treated rats showed a significant motor recovery at a relatively early stage, a small proportion of the transplanted cells integrated at 4 weeks and only a few neurally differentiated cells survived 8 weeks posttransplantation, we propose that the neurotrophic support, especially the high expression of NGF in NPCs, might impact the course of improvement as well as the survival, integration, or myelination of the grafted cells.

In conclusion, neurally induced UCB cells modestly promoted functional recovery in rats with SCI. Therefore, these cells might be a promising stem/progenitor cell source for the repair of damaged spinal cords, potentially extending the use for cell-based therapy in a variety of neurological diseases even though additional long-term studies are required to confirm whether NGF is the major element driving functional effects, and UCB-derived cells necessarily need to be delivered to repair injured spinal cords, for example, comparison between trophic factor alone and UCB-NPCs containing NGF.

Acknowledgements

The authors are grateful to the LifeCord International Co. Ltd. for help in funding. This research was supported by a grant and the program of joint research fellowship from Lifecord Inc., and a grant (SC-4160) from Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

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