Original Research Report

Human Umbilical Cord Blood Stem Cells Infusion in Spinal Cord Injury: Engraftment and Beneficial Influence on Behavior

SAMUEL SAPORTA,1–3 JONG-JOONG KIM,1,5 ALISON E. WILLING,1–3 EUGENE S. FU,1,4 CYNDY D. DAVIS,6 and PAUL R. SANBERG1,3

ABSTRACT

The use of human umbilical cord blood (hUCB)—a rich source of nonembryonic or adult stem cells—has recently been reported to ameliorate behavioral consequences of stroke. In this study, we tested whether human cord blood leukocytes also ameliorate behavioral impairments of spinal cord injury. Rats were divided into five groups: (1) laminectomy (without spinal cord injury) only; (2) laminectomy + cord blood infusion; (3) spinal cord injury + cord blood infused 1 day post injury; (4) spinal cord injury + cord blood infused 5 days post injury; and (5) spinal cord injury only. Spinal cord injury was induced by compressing the spinal cord for 1 min with an aneurysm clip calibrated to a closing pressure of 55 g. Open-field behavior was assessed 1, 2, and 3 weeks after intravenous injection of prelabeled human cord blood cells. Open-field test scores of spinal cord injured rats treated with human cord blood at 5 days were significantly improved as compared to scores of rats similarly injured but treated at day 1 as well as the otherwise untreated injured group. The results suggest that cord blood stem cells are beneficial in reversing the behavioral effects of spinal cord injury, even when infused 5 days after injury. Human cord blood-derived cells were observed in injured areas, but not in noninjured areas, of rat spinal cords, and were never seen in corresponding areas of spinal cord of noninjured animals. The results are consistent with the hypothesis that cord blood-derived stem cells migrate to and participate in the healing of neurological defects caused by traumatic assault.

INTRODUCTION

A number of promising strategies using cell replacement therapy have been proposed for treatment of spinal cord injury. Recent reports have included the use of transplantation of Schwann cell sheaths (1), fetal neurons (2–4), olfactory ensheathing glia (5), neuronal progenitor cells (6), transfected cells that produce growth factors (7), and embryonic stem cells (8) directly into or surrounding the area of damage. Alternative sources of stem cell-like cells may have practical advantages over embryonic stem cells and have been proposed to be used for autologous grafts. Two sources of adult stem cell-like cells that

1Center for Aging and Brain Repair, and Departments of 2Anatomy, 3Neurosurgery, and 4Anesthesiology, University of South Florida College of Medicine, Tampa, FL 33612.
5Chosun University Medical School, Department of Anatomy, Gwang Ju 501-759, Korea.
6Saneron-CCEL Therapeutics, Inc., Tampa, FL 33612.
have received great interest are human stromal bone marrow cells and human umbilical cord blood cells (hUCB) (9–14). We have chosen to concentrate on the use of hUCB cells as a source of stem-like cells that may be useful in the repair of spinal cord injury.

hUCB leukocytes are a heterogeneous population of cells that are enriched in hematopoietic CD34$^+$ stem cells (11,15). However, a small population of CD34$^-$ mononuclear hUCB cells that possess properties of pluripotent stem cells have also been described (9,12,16–17).

The use of hUCB cells has recently been reported to ameliorate behavioral consequences of stroke injury and traumatic brain injury (18,19). One of the more interesting aspects of hUCB cells is their reported ability to target and migrate to areas of damage and engraft within these areas after intravenous infusion (18,19). Such targeted migration would eliminate the need to introduce the cells directly into the central nervous system (CNS). These cells are thereby an attractive source of cells for transplantation because they are readily available, can be expanded, and may be able to target damaged areas of nervous tissue when introduced into the venous circulation.

No reports have appeared using hUCB stem cells for repair of spinal cord injury (SCI). We have examined the ability of hUCB to target a zone of compression injury in the spinal cord following introduction of these cells into the venous circulation 1 or 5 days after SCI, as well as the efficacy of hUCB to ameliorate the behavioral deficits associated with spinal cord compression. The results indicate that cord blood stem cells may provide a useful and novel therapeutic option for patients with spinal cord injury, and emphasize the need for further investigation in this area.

**MATERIALS AND METHODS**

**Animals**

Adult, male Sprague-Dawley rats (Zivic-Miller, Zelienople, PA) were used in these studies. All animals received a laminectomy at T8/9. Rats were randomly assigned to one of five groups: Laminectomy Only (LO); Laminectomy followed by infusion of hUCB 1 day after surgery (L + hUCB); SCI only; SCI followed by infusion of hUCB 1 day (SCI + hUCB1) or 5 days after surgery (SCI + hUCB5). All animals were immunosuppressed with cyclosporine (10 mg/kg per day) for the duration of the experiment and were allowed to survive 3 weeks after injection of hUCB or 4 weeks after laminectomy only or SCI only. Additionally, 6 animals were subjected to a laminectomy and hemicompression to assess the engraftment of hUCB in the spinal cord. Animals were housed under a 12:12 h light–dark cycle and allowed free access to food and water.

**Surgery**

A laminectomy, with removal of the vertebral pedicle, was performed at T8/9 on rats anesthetized with isoflurane. The jaws of a calibrated aneurysm clip with a closing pressure of 55 g were placed between the dorsoventral surfaces of the spinal cord and left in place for 1 min (20). An additional 6 animals received the same laminectomy, but were hemicompressed for 1 min by placing the clip on one side of the spinal cord. All animals were given 0.1 ml of Baytril antibiotic for 5 days following surgery. All rats received appropriate care according to University of South Florida IACUC guidelines and the Principles of Laboratory Animal Care throughout this study. Animals with SCI were individually housed on special bedding to prevent skin breakdown, and had bowel and bladder manual expressed twice daily. Food and water were freely accessible at a lowered height in their cages.

**hUCB cell preparation**

Mononuclear hUCB cells obtained from Saneron-CCEL Therapeutics, Inc. (Tampa, FL) were thawed quickly at 37°C and gently transferred to a 15-cc centrifuge tube filled with 10 ml of Isolyte S, pH 7.4. After thawing, cells were washed, centrifuged at 1000 rpm for 7 min, and placed in media with cholera toxin subunit B conjugated to fluorescein isothiocyanate (CTX-FITC) for 15 min at room temperature to prelabel the cells. The cells were then washed three times. Viability and cell number were assessed and the cells resuspended in media to a concentration of 4000 cells/μl. Viability of hUCB was also determined post-surgically.

**hUCB cell injection**

At 1 or 5 days post-surgery, the rats that were to receive hUCB cells were placed into a restrainer and $1 \times 10^6$ hUCB cells suspended in 0.25 ml of Isolyte were injected intravenously through the tail vein using a 27-gauge needle attached to a 1-ml syringe. Rats with hemicompression injury were injected 24 h following SCI. hUCB cells from one donor were used for infusion in all groups.

**Behavioral testing**

Open-field locomotor activity was scored according to the scale developed by Basso, Beattie, and Bresnahan (21) (BBB). All behavioral tests were digitally videotaped, converted to computer video files, and examined and independently scored by three observers who were unaware of prior treatment. These scores were averaged to arrive at one score for each animal for that behavioral session. All animals were tested at weekly intervals af-
ter surgery or infusion of hUCB cells. Because SCI + hUCB5 or SCI + hUCB1 groups were tested at different times after addition of hUCB cells, an equal number of animals of the LO and SCI groups were randomly distributed and tested with each of these groups.

**Histology**

At the conclusion of behavioral testing, animals were deeply anesthetized with Nembutal (60 mg/kg; Abbott Laboratories), delivered intraperitoneally, and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Spinal cords were removed and preserved frozen in 20% sucrose in 0.1 M phosphate buffer, pH 7.4. Serial 20-μm frozen sections were cut sagittally through the extent of the spinal cord and thaw-mounted onto glass slides to maintain anatomical relationships. Preliminary experiments showed that sections through the area of spinal cord damage contained cells that were endogenously fluorescent (i.e., fluorescent under both the FITC and rhodamine filters). Therefore, sections spaced every 200 μm from all experimental animals were stained with 0.05% Pontamine Sky Blue to suppress nonspecific endogenous fluorescence (22) and examined with fluorescence microscopy to identify prelabeled human cells. Cells that we have identified as hUCB cells were specifically fluorescent under the FITC filter, but not the rhodamine filter. A second series of similarly spaced sections was stained with 1% crystal violet, dehydrated with alcohol and xylene, and examined to define the cytoarchitecture of the spinal cord. Animals with hemicompression of the spinal cord were sacrificed at 1, 2, and 3 days after hUCB injection, and their spinal cords were processed as described above.

**Statistics**

Behavioral data are reported as a mean ± standard error (SE) of each group at each time point and were compared using a two-way analysis of variance (ANOVA) with repeated measures followed by a Newman-Keulls post-hoc analysis (23).

**RESULTS**

Animals of the LO and L + hUCB groups did not demonstrate any signs of motor impairment immediately after recovery from anesthesia. All animals of the SCI groups demonstrated a total paraplegia upon recovery from anesthesia, as well as 2 days after surgery.

**Open-field locomotor behavior**

Open-field behavior over the 3-week post-transplant period is presented in Fig. 1. LO and L + hUCB animals, which never showed any signs of behavioral impairment during the course of the study, scored a perfect 21. Animals with only SCI showed a slight improvement in leg movement over the 3-week test period, with limited hindlimb movement in a few animals, indicating that spinal cord compression did not result in a permanent paraplegia in some of these animals. However, the majority of the SCI-only group of animals did have permanent paraplegia and had a median score below 8 on the BBB 4 weeks after compression injury. ANOVA with repeated measures demonstrated an overall significant effect of hUCB treatment ($F_{11,462} = 875.1, p < 0.0001$). There was a statistically significant effect of treatment ($F_{3,4} = 21.8, p < 0.0001$) and time ($F_{2,3} = 19.6, p < 0.0001$), as well as a significant treatment-by-time interaction ($F_{6,7} = 8.3, p < 0.042$). Post-hoc analyses indicated that locomotor behavior was significantly better in SCI + hUCB5 animals than both SCI + hUCB1 animals ($p < 0.01$) and SCI only animals ($p < 0.01$) at all time points. There was no statistically significant difference between SCI + hUCB1 and SCI only animals at any time point.

**Identification of hUCB cells**

Unambiguous identification of prelabeled hUCB cells in the area of SCI proved difficult due to the high level of endogenous fluorescence present in this injured tissue. To gain a greater understanding of the process of hUCB infiltration into the injured spinal cord, hUCB cells were infused intravenously in animals after hemicompression and the progression of hUCB cell infiltration assessed. Figure 2 shows the progression of hUCB cell engraftment and development of endogenous fluorescence at the
site of injury. One day after injection of hUCB, the area of SCI displayed some endogenous background fluorescence, but CTX-FITC prelabeled hUCB cells were clearly identifiable (Fig. 2A). By 3 days, the endogenous fluorescent background at the area of SCI increased, making absolute identification of CTX-FITC prelabeled hUCB cells difficult. However, Pontamine Sky Blue, which successfully suppressed endogenous fluorescence, improved the identification of CTX-FITC prelabeled hUCB cells dramatically (Fig. 3), allowing unambiguous identification of prelabeled hUCB cells. hUCB cells prelabeled with CTX-FITC were distributed throughout the area of SCI, but were not found in areas of frank necrosis (Fig. 3C). The highest density of CTX-FITC prelabeled hUCB cells was at the area of primary damage, and was gradually reduced as the border with intact spinal
cord tissue was approached. No CTX-FITC prelabeled hUCB cells were found in areas of intact spinal cord (Fig. 3B). No fluorescent hUCB-like cells were visible in these sections. (B) Fluorescent photomicrograph of a section of spinal cord from a laminectomy-only animal that also received an intravenous injection of hUCB cells. No FITC-prelabeled hUCB cells are visible in these sections. (C) FITC-labeled hUCB cells (arrows) in a section from the spinal cord of a rat that received hUCB 5 days after compression injury within the area of compression. (D) Fluorescent photomicrograph through the area of compression of an animal that received hUCB 1 day after compression injury. FITC + hUCB cells similar to those seen in C were seen in these sections (arrows). Calibration bar: A, B, and D, 50 μm; C, 100 μm.

FIG. 3. hUCB cells in the injured spinal cord 4 weeks after intravenous injection. Endogenous fluorescence was quenched with Pontamine Sky Blue (see Materials and Methods). (A) Fluorescent photomicrograph through the area of compression from a SCI-only animal. No fluorescent hUCB-like cells were visible in these sections. (B) Fluorescent photomicrograph of a section of spinal cord from a laminectomy-only animal that also received an intravenous injection of hUCB cells. No FITC-prelabeled hUCB cells are visible in these sections. (C) FITC-labeled hUCB cells (arrows) in a section from the spinal cord of a rat that received hUCB 5 days after compression injury within the area of compression. (D) Fluorescent photomicrograph through the area of compression of an animal that received hUCB 1 day after compression injury. FITC + hUCB cells similar to those seen in C were seen in these sections (arrows). Calibration bar: A, B, and D, 50 μm; C, 100 μm.

cord tissue was approached. No CTX-FITC prelabeled hUCB cells were found in areas of intact spinal cord (Fig. 3B). No similarly fluorescent cells were found in SCI only animals counterstained with Pontamine Sky Blue.

CTX-FITC prelabeled hUCB cells recovered in the spinal cord were usually round or slightly elongated (approximately 10 μm in diameter) with a prominent nucleus. Occasionally, a fusiform FITC-labeled cell was found in the spinal cord within the area of SCI (Fig. 3D). We were uncertain of the quantity of hUCB cells that would enter the spinal cord after intravenous introduction. However, based on the number of sections examined, counting the number of hUCB cells in these sections, and extrapolating to determine a density of cells, we calculate that an average of approximately 370 hUCB cells populated a 14.1-mm³ volume of spinal cord in the SCI + hUCB1, while approximately 870 hUCB cells were found in a similar volume of spinal cord in the SCI + hUCB5 animals. The number of hUCB cells found in the spinal cord in each case was not large, but a greater number of cells were found in the SCI + hUCB5 group than in the SCI + hUCB1 group. The volume of damage produced by the aneurysm clip in each of these groups was similar (SCI + hUCB1 = 42.4 mm³; SCI + hUCB5 = 38.9 mm³).

DISCUSSION

The results of this study demonstrate that hUCB cells introduced intravenously 1 or 5 days after SCI engraft
into an area of SCI and are able to ameliorate some of the behavioral effects of SCI, as measured by spontaneous limb movement in an open-field test, hind limb extension, and toe spread as well as climbing a wire mesh incline. hUCB cells could be found within the area of SCI 4 weeks after their intravenous introduction, but were not present in adjacent, noninjured spinal cord, nor could they be found in normal spinal tissue.

Previously, we have shown that hNT neurons (neurons derived from a human teratocarcinoma cell line) have their greatest behavioral effect and engraft within an area of spinal cord contusion when transplanted 2 weeks after SCI, rather than immediately (6). We hypothesized that transplants survive better within the parenchyma when cytokine levels, which increase dramatically immediately after SCI, begin to subside (see ref. 24). This hypothesis has recently been strengthened by research confirming that, at least for traumatic injury of the CNS, delaying transplant until inflammatory cytokines begin to subside results in better engraftment of the transplant and improved behavioral outcome (2,25).

The intravenous introduction of hUCB cells has distinct advantages over the more conventional direct transplantation of cells into the area of damage. Whereas direct transplant of hUCB cells produces a high density of therapeutic cells at the site of introduction, these cells do not migrate far from the transplant site. The distribution of hUCB cells after intravenous injection was sparse in the spinal cord, but hUCB cells were found throughout the area of direct SCI and most of the area of secondary damage. Few hUCB cells were found at the margin between intact and injured spinal cord, and no hUCB cells were ever found in intact areas of spinal cord. It may be that hUCB cells enter those areas that are directly damaged by SCI through damaged blood vessels at the site of injury and/or through a compromised blood–brain barrier in areas of secondary damage.

Studies that examined the efficacy of hUCB cells in an in vivo model of stroke also reported that hUCB cells engraft into the area of ischemic injury and differentiate into cells with astrocytic and neuronal phenotypes (9,19). Our goal in this study was to examine whether there was an amelioration of behavioral deficits induced by SCI. Having concluded that there is an efficacious effect, the characteristics of the hUCB cells recovered in injured spinal cord now become a question of great interest. We suspect that some of the hUCB cells recovered in the spinal cord of the present study would be glial, and a few would demonstrate a neuronal phenotype, as was found in previous studies (9,19). However, the morphology of the recovered hUCB cells that were seen in the spinal cord was not characteristically astrocytic or neuronal. It may be that the entire population of hUCB cells that engraft in spinal cord does not differentiate into an astrocytic or neuronal phenotype. Additionally, it seems highly unlikely that the presence of fewer than 1000 hUCB cells within the area of damaged spinal cord would be sufficient to restore motor function. It may be that the release of trophic factors by hUCB cells may be sufficient to support damaged tissue, which may subsequently allow behavioral recovery (18). We are presently addressing this question for spinal cord in a more extensive series of studies focused on comparing in vivo engraftment and differentiation of hUCB cells transplanted directly into the damaged spinal cord to hUCB cells that populate the area of SCI after being injected intravenously.

The presence of stem cells from nonembryonic tissues has promoted interest in using stem cells to repair damage in the nervous system. Although previously thought to produce only cells of the organ in which they were found, certain adult stem cells appear to have a more multipotential capacity (26). For example, bone marrow stem cells and hUCB cells can be differentiated to express neuronal and glial phenotypes in vitro (12,17,27). Interestingly, hUCB stem cells, which have been shown to be capable of producing neuron-like cells, have also been used to repopulate bone marrow (28), and both bone marrow stem cells, as well as hUCB cells, are able to adopt a neuronal or glial phenotype, implying that hemopoiesis and neuropoiesis may share a number of common elements (9,13,14,17,28,29). Migration and differentiation of bone marrow–derived neural cells within the central nervous system has also been reported (13). Moreover, human CNS stem cells form neurospheres in culture and can differentiate into neurons and glial cells when treated with growth factors (24,30). When transplanted intraventricularly into neonatal immunodeficient mice, these cells migrate, proliferate, and differentiate into neurons and astrocytes that are CD133+, CD45−, and CD34−, a phenotype similar to that of certain stem cells found in hUCB cells (24,31–33). Thus, hUCB may be a viable source of stem cells for treatment of neurological disorders. Further study is needed to validate this hypothesis and characterize the cells involved in the therapeutic benefits observed in this report.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Lucy Colina, Department of Anatomy, and Christopher Jackson, Department of Anesthesiology. This work was supported by the Florida Spinal Cord Injury Program. Dr. Kim was a Visiting Scholar supported, in part, by Chosun University. Drs. Saporta and Willing are consultants for, and Dr. Sanberg a founder of, Saneron-CCell Therapeutics, Inc.
REFERENCES


Address reprint requests to:
Dr. Samuel Saporta
Department of Anatomy
College of Medicine
University of South Florida Health Sciences Center
12901 Bruce B. Downs Boulevard
Tampa, FL 33612

E-mail: ssaporta@hsc.usf.edu

Received March 16, 2003; accepted March 18, 2003.