Central Nervous System Entry of Peripherally Injected Umbilical Cord Blood Cells Is Not Required for Neuroprotection in Stroke

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Background and Purpose—To date, stem cell graft-mediated neuroprotection is equated with graft survival and secretion of neurotrophic factors in the brain. Here, we examined whether neuroprotection by systemically delivered human umbilical cord blood (HUCB) cells was dependent on their entry into the central nervous system in a rodent model of acute stroke.

Methods—Adult male Sprague-Dawley rats were subjected to right middle cerebral artery occlusion for 60 minutes. During the 1-hour occlusion, animals were randomly assigned to 1 of the following treatments: intravenous injection of HUCB (a subtherapeutic dose of 200 000 cells in 10 μL) with blood–brain barrier (BBB) permeabilizer (1.1 mol/L mannitol at 4°C) or vehicle, intravenous vehicle alone, or intravenous mannitol alone. Behavioral tests, using elevated body swing test and passive avoidance test, were conducted at day 3 poststroke, and thereafter, animals were euthanized for: (1) immunohistochemical examination of HUCB, which were lentivirally labeled with green fluorescent protein; (2) cerebral infarction analysis using 2,3,5-triphenyl-tetrazolium chloride; and (3) enzyme-linked immunosorbent assay of trophic factors within the striatal region.

Results—We did not detect intravenously administered low dose of HUCB cells in the brains of animals at day 3 after stroke even when cells were coinfused with a BBB permeabilizer (mannitol). However, HUCB–mannitol treatment significantly increased brain levels of neurotrophic factors, which correlated positively with reduced cerebral infarcts and improved behavioral functions.

Conclusions—Our data show that central nervous system availability of grafted cells is not a prerequisite for acute neuroprotection provided that therapeutic molecules secreted by these cells could cross the BBB. (***Stroke***. 2004;35: 2385-2389.)

Key Words: blood–brain barrier ■ cell transplantation ■ cerebral infarction ■ cerebral ischemia ■ stem cells

Neuroprotection produced by stem cells has recently come under intense scientific and public scrutiny.[1–3] New clinical evidence reveals that peripherally injected human umbilical cord blood (HUCB) cells do not generate new nerve cells in the brain of a deceased patient.[4] Laboratory data also indicate failure of hematopoietic bone marrow cells to differentiate into brain cells.[5,6] However, equally compelling evidence demonstrates that peripherally transplanted adult human bone marrow cells in patients with hematologic malignancies entered the brain and generated neurons.[7,8]

Neural transplantation has been shown to ameliorate functional and cognitive deficits in animal models of neurological disorders and has reached clinical trials in Parkinson disease, Huntington disease, and stroke.[9–11] The ultimate goal of cell therapy is to deliver viable cells into the injured brain with the hope that these grafted cells or the neurotrophic factors they released will re-establish the damaged host neural connections, either by forming new networks or reconstructing the old pathways.

Accordingly, the yardstick for evaluating a successful transplantation outcome is visualization of graft survival in the brain. Indeed, absence of surviving grafts correlates with continued display of neurological deficits in transplant recipients in animal models of central nervous system (CNS) disorders and patients.[12,13] These findings largely formed the scientific bias for dismissing transplantation results as "placebo effects" when graft survival could not be unequivocally demonstrated in the event of functional recovery. The present study now reveals that transplant-mediated functional recovery could occur in stroke without CNS entry of grafted cells

provided that neuroprotective molecules secreted by these cells crossed the blood–brain barrier (BBB) and reached the injured brain site.

Materials and Methods

Animals

We examined the histological and neurobehavioral effects of early intravenous delivery of HUCB cells into rats after transient middle cerebral artery (MCA) occlusion. Adult Sprague-Dawley, male rats (Harlan, Indianapolis, Ind) weighing 250 to 300 g served as subjects in this study. National Institutes of Health and institutional animal care and use committee guidelines for use of animals in research were followed. Animals were subjected to right MCA occlusion (MCAo) for 1 hour, then randomly assigned to 1 of the following treatments: intravenous injection of HUCB (200 000 cells in 10 μL) with BBB permeabilizer (1.1 mol/L mannitol at 4°C; n=9) or vehicle (PBS; n=10), intravenous vehicle alone (n=8), or intravenous mannitol alone (n=8). An additional weight-matched group of adult male Sprague-Dawley rats (n=10) served as positive controls (ie, nonstroke, nontransplanted, no drug treatment). Behavioral tests[12,14] were conducted at day 3 after stroke, and thereafter, animals were euthanized for histological analysis of HUCB cell survival. To reveal HUCB effects on cerebral infarction, a new set of animals (n=10 per group) was subjected to the same treatment.

Stroke Surgery

MCAo stroke surgery followed the procedures described in detail previously.[12,15,16] Anesthetized (equithesin 300 mg/kg IP) animals were subjected to this well-established suture technique that occludes the right MCA for 1 hour. A heating pad and a rectal thermometer allowed maintenance of body temperature at normal limits. To ensure similar degree of stroke insults, physiological parameters including PaO2, PaCo2, and plasma pH measurements were monitored in each animal, and we found no significant differences in animals used in this study. In addition, to ensure successful arterial occlusion, we used a laser Doppler (Perimed 5001) to monitor cerebral blood flow and obtained ≥80% reduction in cerebral blood flow during MCAo in all the animals used here. Mean body weights of stroke animals did not significantly differ across treatment groups over the 3-day poststroke period.

HUCB Lentiviral Green Fluorescent Protein Labeling

HUCB cells were prelabeled with green fluorescent protein (GFP) using lentiviral vector strategy[17,18] to allow visualization of grafted cells. We have successfully grown in our laboratory lentiviruses supplied by Dr Didier Trono (University of Geneva, Geneva, Switzerland). Our pilot data demonstrate that this strategy is highly effective in producing infectious viral particles expressing GFP (>75%) in cultured and grafted HUCB cells.

HUCB Cell Transplantation

During the 1-hour occlusion of the MCA, anesthetized (equithesin, 300 mg/kg IP) animals received intravenous (jugular vein) injection of HUCB (200 000 cells from Saneron CCEL Therapeutics, Inc, Tampa, Fla, in 10 μL PBS) or vehicle (PBS, same volume) over 10 minutes. Immediately thereafter, using the same intravenous line, animals received either 1.1 mol/L mannitol (maintained at 4°C) or vehicle (PBS, also maintained at 4°C) over 5 minutes. For trophic factor neutralization experiments, HUCB cells were exposed before transplantation to established neutralizing concentrations of specific trophic factors antibodies (R&D Systems) as described previously.[19,20]

Behavioral Testing

Motor and cognitive deficits were characterized at day 3 after stroke to allow a stringent analysis of neuroprotection at a time point of maximal infarction. To reveal motor asymmetry, animals were subjected to the elevated body swing test (EBST).[12,14]

We have used the EBST previously and noted that MCAo stroke animals display >75% biased swing activity as early as the day of stroke surgery and remain stable for ≤2 months.[12,14] To reveal cognitive performance, animals were also introduced to passive avoidance testing as described in detail previously.[12,14] Acquisition of the task was measured in terms of the amount of time it took the rat to remain on the platform continuously for 3 minutes. A retention test was conducted by placing the rat on the platform exactly as before and recording the latency to step-down measured to a maximum of 3 minutes. MCAo stroke animals display significant impairments in acquisition and retention of the task as early as 24 hours after ischemia, which persist at ≈6 months after ischemia.[12,14]

GFP Epifluorescence Microscopy and Immunohistochemistry

HUCB cell graft survival was examined initially using GFP epifluorescence (fluorescein isothiocyanate fluorochrome). Because not all HUCB cells were GFP labeled, we also conducted immunohistochemistry using a human specific antibody 11 to reveal GFP-negative HUCB cells. Animals were anesthetized with xylazine (13 mg/kg IP) and ketamine (44 mg/kg IP) then perfused with saline (150 mL) via a cardiac catheter. The brain was removed and stored in 4% paraformaldehyde with 25% sucrose until cryostat sectioning. The whole brain was cut, and all 20-μm cryostat sections were processed for immunohistochemistry using standard avidin biotinylated enzyme complex method.[12] A monoclonal antibody MOC-1 that recognizes a human-specific epitope in neural cell adhesion molecule (N-CAM) and does not cross-react with rodent N-CAM or other rodent proteins was used to detect grafted HUCB cells in the rat brain. All brain sections were examined using a Zeiss Axiophot 2 microscope at ×20 and ×40 magnifications.

Cerebral Infarct Analysis

Using an NIH imaging system, the brain tissues were processed for triphenyltetrazolium chloride (TTC) staining to measure stroke volumes using the following formula: 20 μm (thickness of the slice)×[sum of the infarction area in all brain slices (um^2)].[21]

Enzyme-Linked Immunosorbent Assay

We followed the ELISA method described previously[22] with minor modifications. Trophic factor antibodies were obtained from R&D Systems.

Statistical Analysis

Behavioral scores and infarct volumes were initially analyzed using ANOVA, followed by post hoc Bonferroni t tests for pairwise comparisons between treatment groups. The level of significance was set at <0.05.

Results

HUCB Grafts Improve Motor and Cognitive Performance

Previous studies on the effects of HUCB in stroke[23,24] demonstrated that behavioral recovery does not occur immediately but rather over a protracted period of time (ie, several weeks). In the present study, behavioral tests of motor and cognitive function were conducted at a single early time point after stroke (ie, day 3) to determine whether combining HUCB with mannitol would produce more immediate and robust effects. Although HUCB plus vehicle was ineffective on all tests at this early time point (ie, no benefits relative to animals receiving stroke plus mannitol or vehicle only), combining HUCB cells with mannitol produced an impressive profile of behavioral recovery. Stroke-induced motor deficits, measured by percent motor asymmetry using EBST,
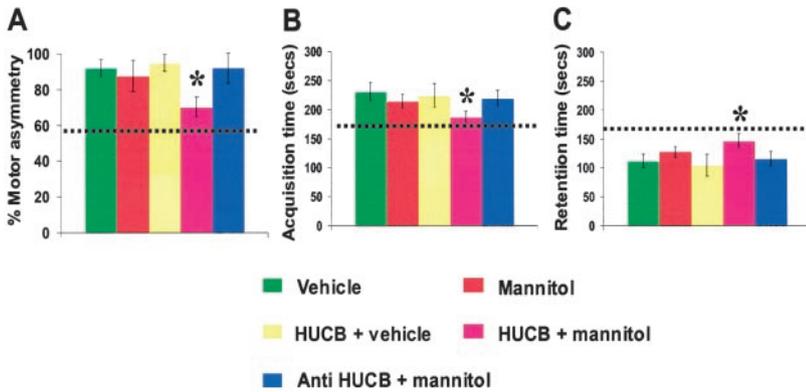
Figure 1. Stroke animals treated with intravenous HUCB + mannitol displayed significantly reduced motor asymmetry (A) in the EBST and decreased acquisition time (B) and increased retention time (C) in the passive avoidance task at day 3 after stroke compared with animals treated with intravenous vehicle alone (Vehicle), intravenous mannitol alone (Mannitol), or intravenous HUCB + vehicle (*t tests; $P<0.05$). However, they remained partially impaired compared with control animals (nonstroke, nontransplanted animals; dotted lines). Pretreatment of HUCB with neutralizing antibodies against GDNF, NGF, and BDNF (Anti HUCB + mannitol) blocked neuroprotection.

were significantly reduced by 22% when HUCB cells were combined with mannitol ($F_{(5,59)}=91.57$; $P<0.0001$; Figure 1A). Cognitive deficits measured by learning a passive avoidance task showed a significant 16% reduction ($F_{(5,59)}=6.93$; $P<0.0001$) in acquisition time (Figure 1B), and an analysis of memory of this same task revealed a significant 28% increase ($F_{(5,59)}=30.11$; $P<0.0001$) in retention time (Figure 1C).

HUCB Grafts Reduce Cerebral Infarcts

Although behavioral recovery has been reported consistently using HUCB cells in stroke animals,[23,24] no report has yet demonstrated that HUCB transplants are capable of reducing infarct volume in those same animals. Here we conducted TTC staining at day 3 after stroke when MCAo infarction was maximal. Data revealed that intravenous HUCB limited the infarct volume (40% reduction) but only when combined with mannitol ($F_{(5,59)}=335.64$; $P<0.0001$; Figure 2). Compared with the established antiedema regimen of 20% mannitol delivered at 2.5 g/kg bolus every 6 hours,[25] the present acute mannitol regimen did not reduce edema in our stroke animals.

Absence of Detectable HUCB Grafts in Stroke Brain

Immunohistochemical and GFP epifluorescence microscopy revealed no detectable intravenous HUCB cells in the brains of all animals at day 3 after stroke. To eliminate the possibility that lentivirally tagged HUCB might have lost

their human phenotype and GFP labeling after transplantation, parallel studies exposed stroke animals (n=20) to stereotaxic delivery of HUCB into the striatum, and we found that intraparenchymally grafted HUCB cells were positively labeled with human-specific N-CAM and GFP (Figure 3), which persisted over long-term post-transplantation (ie, hours to 6 months of graft maturation). To determine whether HUCB cells might have entered at earlier periods after stroke and died during the disease progression, additional animals (n=30) were subjected to MCAo and received either intravenous HUCB plus vehicle or intravenous HUCB plus mannitol and were randomly euthanized at 1, 2, 4, 8, 24, and 48 hours after stroke. These additional studies also revealed no detectable intravenous HUCB cells in the brain. Together, these results confirmed that peripherally administered HUCB cells did not cross the BBB with or without mannitol treatment.

HUCB Grafts in Peripheral Organs

Histologic examination of systemic organs at day 3 after stroke revealed some human N-CAM–positive cells in kidneys, lungs, and spleens but not in the livers and hearts in transplant recipients of intravenous HUCB with or without mannitol (graft survival not significantly different between these 2 groups). Thus, mere survival of HUCB cells in the peripheral organs of animals that did not receive mannitol was not enough to produce neuroprotection. Hematoxylin/eosin staining did not detect any tissue damage and tumor formation.
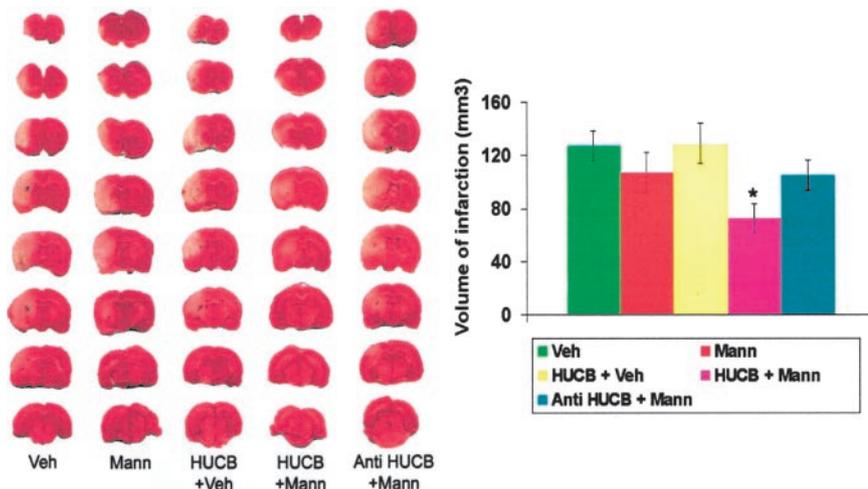

Figure 2. TTC staining revealed that intravenous HUCB + mannitol (HUCB + Mann) significantly decreased stroke volumes compared with animals treated with intravenous vehicle alone (Veh), intravenous mannitol alone (Mann), or intravenous HUCB + vehicle (HUCB + Veh; *t tests; $P<0.05$). Pretreatment of HUCB with neutralizing antibodies (Anti HUCB + Mann) prevented neuroprotection.
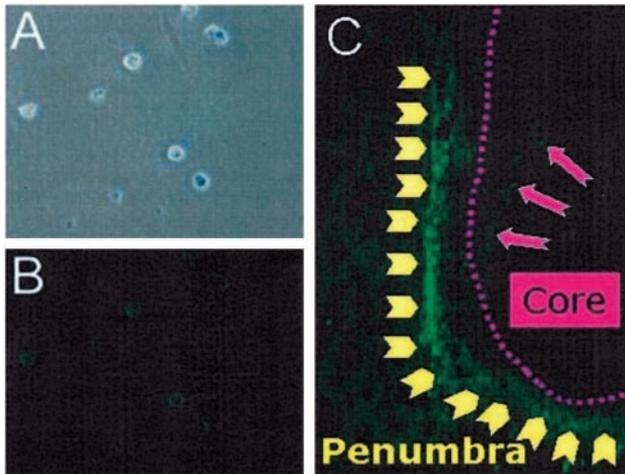
Figure 3. Detection of HUCB cells in the brain. GFP labeling of HUCB cells using lentiviral vector strategy revealed good transduction in vitro (A, phase contrast; B, GFP), which remained stable after stereotaxic transplantation into the striatum (day 3 after stroke shown in C). However, intravenously delivered HUCB cells could not be detected in the brain with or without mannitol.

Trophic Factor Mediation in HUCB Graft Neuroprotection

In the absence of detectable HUCB cells in the brain, we hypothesized that HUCB cells either exerted a direct trophic effect on the damaged tissue or elevated endogenous levels of trophic activity. Separate sets of identically treated animals (n=6 per group: stroke then intravenous HUCB plus vehicle, intravenous HUCB plus mannitol, intravenous mannitol alone, or intravenous vehicle alone) were used to measure brain levels of glial cell line–derived neurotrophic factor (GDNF), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) using ELISA.[22,26] Replicating the above data, these separate studies again confirmed that combining intravenous HUCB cells with mannitol produced significant behavioral and histological protective effects (data not shown). These studies also revealed associated elevations of brain levels of GDNF (68% above controls; Figure 4) at day 3 after stroke.

Additional ELISA studies at days 1 and 2 after stroke, designed to determine the timing of elevations of trophic
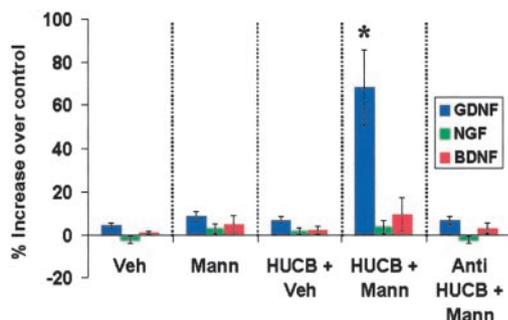

Figure 4. ELISA revealed that intravenous HUCB + mannitol (HUCB + Mann) increased GDNF brain levels at day 3 after stroke compared with animals treated with intravenous vehicle alone (Veh), intravenous mannitol alone (Mann), or intravenous HUCB + vehicle (HUCB + Veh; *t* tests; $P<0.05$). Pre-exposure of HUCB cells to neutralizing trophic factor antibodies prevented increments in GDNF brain levels.

factors in stroke animals that received intravenous HUCB and mannitol, revealed increased brain levels of all 2 trophic factors at day 1, but only GDNF was sustained on subsequent days 2 and 3 after stroke (data not shown). These results parallel studies demonstrating that intracerebral GDNF application protects against stroke.[15,21]

To further confirm the potential involvement of trophic factors in the observed neuroprotection, 1 set of animals received MCAo plus intravenous HUCB and mannitol (n=12), whereas a second group (n=20) of stroke animals was treated identically except that HUCB cells were exposed to antibodies against GDNF, NGF, and BDNF before transplantation. As anticipated, elevations in GDNF levels were prevented (Figure 4), and the behavioral (Figure 1) and histological (Figure 2) protective effects were completely blocked by pretreatment of HUCB cells with antibodies to trophic factors.

Finally, we measured blood levels of GDNF, NGF, and BDNF to further determine the source of trophic factors. Additional stroke animals (n=6 per group) were subjected to the same surgical/drug regimen as above and euthanized at day 3 after stroke. ELISA revealed low (15% above controls) but detectable levels of trophic factors from circulating blood in stroke animals that received intravenous HUCB grafts plus mannitol. In contrast, no detectable levels of trophic factors were obtained from stroke animals injected either with intravenous HUCB grafts alone, mannitol alone, or vehicle alone. We assayed subsequently the peripheral organs (kidneys, lungs, and spleens), where we found GFP-labeled HUCB grafts, and detected significant elevations (125% to 160% above controls) in trophic factors in stroke animals that received either the HUCB grafts plus mannitol or HUCB grafts alone compared with those that received mannitol alone or vehicle alone (nonsignificant increment of 8% above controls).

Discussion

Intravenous delivery of HUCB when combined with mannitol promoted neuroprotection with the present low dose of 200 000 HUCB cells, which mimics those seen in other reports using much higher doses (>500 000 cells) of intravenous HUCB.[23,24] In addition, whereas intravenous HUCB alone shows therapeutic effects when administered 24 hours to 7 days after injury,[23,24,27] the addition of mannitol, which is used clinically for hyperosmolar therapy, allowed for the cells to be injected within minutes after stroke.

Although the delay in intravenous HUCB transplantation led to grafted cell visualization in the brain,[23,24] whereas the present early intravenous HUCB plus mannitol did not, both strategies produced neuroprotection. The advantage of early delivery of cells after stroke with adjunct mannitol is that an enhanced therapeutic effect (ie, reduced cerebral infarction) was achieved. Of note, the present acute neuroprotection was induced without immunosuppression, which is generally requisite for long-term graft survival and often accompanied by deleterious side effects.

A major finding here is that spontaneous BBB opening produced by MCAo was not permissive enough to allow CNS entry of endogenous or graft-derived trophic factors, suggesting the need for exogenous BBB manipulation. Whereas trophic factors were detected in peripheral organs of stroke animals that

received HUCB grafts alone, mannitol alone, or vehicle alone, a perturbed BBB permeability produced by mannitol was shown critical for mobilizing graft-derived trophic factors to be present in the circulating blood, as well as the brain to exert neuroprotection. The lack of significant increments in blood and brain trophic factor levels in stroke animals that received mannitol alone suggests that HUCB grafts, instead of the host tissues per se, were likely the source of neuroprotective trophic factors. Moreover, the critical timing of mannitol-facilitated entry of trophic factors into the stroke brain is exemplified in our neutralization studies. HUCB cells rendered biologically inert (ie, not able to secrete trophic factors) during mannitol activation remained viable in the periphery and probably resumed secretion of trophic factors after the neutralizing effects had waned and when spontaneous BBB opening or cell migratory factors had peaked, but such delay in trophic factor secretion did not lead to neuroprotection. Because endogenous cell migratory factors are elevated in ischemic brain areas at >1 day after focal stroke,[28] CNS recruitment of peripherally administered cells may benefit from delayed transplantation. However, in view of abrupt and deleterious pathologic consequences inherent in stroke, the robust functional outcome with early HUCB grafts combined with mannitol may prove more beneficial than a delay transplant regimen.

In summary, neuroprotection in acute stroke was induced by HUCB transplantation from the periphery via BBB permeabilization. Intravenous delivery of HUCB poses as an efficient and noninvasive cell therapy for CNS disorders characterized by a narrow therapeutic window. A multidrug treatment for stroke may be realized via a cell-based therapy that involves routine clinical intravenous infusion of stem/progenitor cells, allowing the biological release of a cocktail of trophic factors into the brain. In the end, our study shows that in addition to detecting grafted cells, trophic factor elevation in the brain is a major index of transplant-induced neuroprotection.

Acknowledgments

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