Infusion of Human Umbilical Cord Blood Cells in a Rat Model of Stroke Dose-Dependently Rescues Behavioral Deficits and Reduces Infarct Volume

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Background and Purpose—Intravenously delivered human umbilical cord blood cells (HUCBC) have been previously shown to improve functional recovery of stroked rats. To extend these findings, we examined the behavioral recovery and stroke infarct volume in the presence of increasing doses of HUCBC after permanent middle cerebral artery occlusion (MCAO).

Methods—Rats were subjected to MCAO and allowed to recover for 24 hours before intravenous infusion of $10^4$ up to $3 \times 10^7$ HUCBC. Behavioral tests (spontaneous activity, step test, elevated body swing test) were performed 1 week before MCAO and at 2 and 4 weeks after HUCBC infusion. On completion of behavioral testing, animals were euthanized and brain infarct volumes quantified. HUCBC were identified by immunofluorescence for human nuclei and by polymerase chain reaction (PCR) using primers specific for human glycerol 3-phosphate dehydrogenase.

Results—At 4 weeks after infusion, there was a significant recovery in behavioral performance when $10^6$ or more HUCBC were delivered ($p < 0.001$ to $p < 0.05$). Infarct volume measurements revealed an inverse relationship between HUCBC dose and damage volume, which reached significance at the higher HUCBC doses ($10^7$ cells, $p < 0.01$; $3 \times 10^7$ cells, $p < 0.05$). Moreover, HUCBC were localized by immunohistochemistry and PCR analysis only in the injured brain hemisphere and spleen.

Conclusions—These results extend previous observations of HUCBC infusion in the MCAO rat stroke model by demonstrating a dose relationship between HUCBC, behavioral improvement, and neuronal sparing. (Stroke. 2004;35:2390-2395.)

Key Words: acute stroke • cell transplantation • neuroprotection

Although using embryonic, fetal, and adult brain-derived neural stem cells may be a viable approach for treatment of neurological disease,1,2 ethical and moral concerns, as well as limited availability, have prompted the search for alternative stem cell sources. Stem cells harvested from bone marrow and cord blood can exhibit neuronal or glial cell properties under defined culture conditions.3-5 Moreover, these cells can mediate therapeutic effects in several animal models of neurological diseases, including stroke.6-9 Cord blood has emerged as an alternative to bone marrow because of its greater availability, weak immunogenicity, and lower risk of mediating viral transmission.10 We have reported significant behavioral benefit after intravenous infusion of human umbilical cord blood cells (HUCBC) in the rat middle cerebral artery occlusion (MCAO) model of stroke.8,11 Moreover, we demonstrated that intravenously infused HUCBC was comparable to, if not better than, intrastriatally transplanted HUCBC in mediating behavioral recovery after MCAO.8

Because a specific dose range has not been defined, the present study characterizes the best dose at which intravenously delivered HUCBC mediate neurological recovery and limit brain damage after stroke injury in adult rats.

Materials and Methods

Animals

Adult Sprague Dawley rats (average body weight range, 175.0±25.0 grams; Harlan, Indianapolis, Ind) were housed in a temperature-controlled room with water and chow ad libitum. Rats were randomly assigned to 7 groups: sham surgery (n=4), MCAO only (n=13), rats infused with $10^4$ (n=6), $10^5$ (n=6), $10^6$ (n=6), $10^7$ (n=6), or 3 to $5 \times 10^7$ (n=4) HUCBC 24 hours after MCAO.
The rats were anesthetized with isoflurane (2% to 5% in O₂ at 2 L/min). The right common carotid artery and external carotid artery were exposed and an embolus (4.0 monofilament) was inserted through the external carotid to the origin of the MCA. The embolus was tied-in permanently. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of South Florida in accordance with the National Institutes of Health guidelines.

Preparation of HUCBC for Transplantation
The HUCBC were donated for research purposes from a cord blood cell bank (Saneron CCEL Therapeutics, Inc, Tampa, Fla). Cryopreserved HUCBC were rapidly thawed at 37°C and resuspended into Isolyte S, pH 7.4 (BBraun/McGaw Pharmaceuticals). Viability and cell quantification was determined by the trypan-blue dye exclusion method.

Transplantation of HUCBC
The animals were anesthetized with isoflurane and the femoral vein was isolated. Cells were delivered with a 26-gauge Hamilton syringe in a volume of 500 μL over 5 minutes. The MCAO-only rats received the HUCBC-free media (Isolyte S). After the infusion, all animals were injected with cyclosporin A daily (10 mg/kg intraperitoneally). Because cyclosporin has neuroprotective activity in MCAO, animals that did not receive HUCBC were also injected daily with cyclosporin A.

Behavioral Measurements
Behavioral tests were performed in all animals before MCAO, and at 2 and 4 weeks after MCAO.

Spontaneous Activity
Spontaneous activity was measured by the automated VersaMax System (Accuscan Instruments, Inc) as previously described. Thirteen locomotor parameters (Table 1) were measured every 5 minutes for 1 hour, and the total activity for each parameter was calculated as the sum of all the 12 measures.

Elevated Body Swing Test
Rats were examined for lateral movements after elevation by their tails to ~10 cm above the surface of the testing area.

TABLE 1. Overall ANOVA Significance (p) for Spontaneous Activity

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group × Time Post Infusion</th>
<th>Group × Observational Period</th>
<th>Group × Time Post Infusion × Observational Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counter-clockwise rotation</td>
<td>0.003</td>
<td>0.0902</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Center time</td>
<td>0.12</td>
<td>0.198</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Center distance</td>
<td>0.077</td>
<td>0.965</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Clockwise rotation</td>
<td>0.002</td>
<td>0.127</td>
<td>0.150</td>
</tr>
<tr>
<td>Horizontal activity</td>
<td>0.025</td>
<td>0.025</td>
<td>0.33</td>
</tr>
<tr>
<td>Movement number</td>
<td>0.171</td>
<td>0.001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Movement time</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Stereotypy counts</td>
<td>0.015</td>
<td>0.663</td>
<td>0.001</td>
</tr>
<tr>
<td>Stereotypy number</td>
<td>0.04</td>
<td>0.0001</td>
<td>0.001</td>
</tr>
<tr>
<td>Stereotypy time</td>
<td>0.030</td>
<td>0.598</td>
<td>0.001</td>
</tr>
<tr>
<td>Total distance</td>
<td>0.14</td>
<td>0.328</td>
<td>0.001</td>
</tr>
<tr>
<td>Vertical activity</td>
<td>0.027</td>
<td>0.127</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vertical movements</td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

TABLE 2. Post Hoc Statistical Comparison (p) for Spontaneous Activity

<table>
<thead>
<tr>
<th>Variable</th>
<th>Time of Analysis</th>
<th>Significance (p) Between the Media-Treated and HUCBC-Treated Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Counter-clockwise rotation</td>
<td>2 wk</td>
<td>10⁰ (p=0.03), 10⁴ (p=0.005), 3·5×10⁷ (p=0.04)</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>10⁰ (p=0.008), 10⁴ (p=0.003), 3·5×10⁷ (p=0.02)</td>
</tr>
<tr>
<td>Clockwise rotation</td>
<td>2 and 4 wk</td>
<td>10⁰ (p=0.046), 10⁰ (p=0.005)</td>
</tr>
<tr>
<td>Horizontal activity</td>
<td>2 wk</td>
<td>10⁰ (p=0.05), 10⁴ (p=0.05), 3·5×10⁷ (p=0.05)</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>10⁰ (p=0.05), 10⁴ (p=0.05)</td>
</tr>
<tr>
<td>Movement time</td>
<td>2 wk</td>
<td>10⁰ (p=0.009), 10⁴ (p=0.043), 10⁰ (p=0.020)</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>10⁰ (p=0.05)</td>
</tr>
<tr>
<td>Stereotypy counts</td>
<td>2 wk</td>
<td>10⁰ (p=0.04)</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>NS</td>
</tr>
<tr>
<td>Total distance</td>
<td>2 and 4 wk</td>
<td>10⁰ (p=0.04), 10⁰ (p=0.04), 10⁰ (p=0.001), 10⁰ (p=0.046), 3·5×10⁷ (p=0.05)</td>
</tr>
<tr>
<td>Vertical activity</td>
<td>2 and 4 wk</td>
<td>10⁰ (p=0.002)</td>
</tr>
<tr>
<td>Vertical movements</td>
<td>2 wk</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>4 wk</td>
<td>Between 10⁰ and 3·5×10⁷ (p=0.010)</td>
</tr>
</tbody>
</table>
of left or right swing was scored over 20 consecutive trials. The bias was calculated as the absolute value of the difference between the number of swings on the impaired side minus 10.

**Step Test**
Rats were held tightly with one forelimb on a hard flat surface and subsequently pulled for a length of 1 meter in the direction of the placed forelimb. The difference between number of steps performed with the right paw and the left paw was calculated.

**Pathological Evaluation**
At the conclusion of the behavioral tests, rats were anesthetized with Nembutal (150 mg/kg, intraperitoneally) and perfused transcardially with 0.1 mol/L phosphate buffer, followed by 4% paraformaldehyde. Brains and other organs (heart, lungs, liver, kidney, spleen, thymus, and bone marrow) were harvested, cryoprotected in a 20% sucrose/0.1 mol/L phosphate buffer solution, followed by cryosectioning (20 μm). The Nissl–Thionine staining was performed by application of the Nissl–Thionine dye for 50 seconds. The areas of cerebral infarction were delineated at 6 preselected coronal sections (from 1.7 mm anterior to bregma through 3.3 mm posterior to bregma) and quantified using an image analyzer (Image Pro Plus). The total volume of ischemic tissue and that of the contralateral hemisphere were calculated as follows: total volume = Σ(area of predefined coronal sections mm³) × intersection distance (mm). Because at 1 month after MCAO the injured brain hemisphere has typically undergone a shrinkage process, we calculated the volume of ischemic damage as percent of contralateral (intact) hemisphere volume.13

**Polymerase Chain Reaction Analysis**
DNA was obtained from the brain and other organs of half of the animals for each group. Polymerase chain reaction (PCR) was performed for the human glycerol-3-phosphate dehydrogenase (HG3PDH) gene using primers (sense: 5′-GGCTGGGACTCATGGAGAT-3′; and antisense: 5′-GGGGTAAGTCGTTGA-GAAAG-3′). Nested PCR was performed with primers (sense: 5′-TCCTGGAGACGTGGTTGTTG-3′; antisense: 5′-GTACCGAAAAGCTGCG-3′). Products were resolved on 3.5% polyacrylamide gels and visualized by silver staining.

**Immunohistochemistry**
Autofluorescence was first quenched using the method of Steele et al.,14 after which the sections were incubated with mouse monoclonal antibody against human nuclei (HuNu; Chemicon, Inc), followed by fluorescein isothiocyanate conjugated goat antimouse secondary antibody (Alexa Molecular Probes). DAPI staining (Molecular Probes, Eugene, Ore) was performed to visualize nucleated cells. Slides were examined under epifluorescence on an Olympus BX60 microscope.

**Statistical Analysis**
The behavioral data were analyzed using ANOVA with repeated measures and the Kruskall–Wallis H test was used for post hoc comparisons. Infarct size was analyzed with ANOVA and the Kruskall–Wallis H test were performed (Table 2).

**Results**

**HUCBC Dose-Dependent Reduction of MCAO-Associated Asymmetry and Hyperactivity: Spontaneous Activity**
For 10 of the 13 analyzed parameters, the overall ANOVA revealed significant differences between the groups (p<0.0001 to p<0.04). Moreover, on 9 of the 13 variables there were significant interactions between groups and time postinfusion, groups and observational period, and groups, time postinfusion, and observational period (p<0.001 to p<0.03) (Table 1). When significant differences were found in the overall analysis, post hoc comparisons with the Kruskall–Wallis H test were performed (Table 2).

For the parameters counter-clockwise rotations, horizontal activity, movement time, stereotypy count, stereotypy number, stereotypy time, and vertical movement number differences between the dose groups were examined separately at 2 and 4 weeks after MCAO. On counter-clockwise rotations, at 2 weeks, the animals treated with 10⁴, 10⁵, and 3 to 5×10⁷ cells were significantly less active than the media-treated controls (p=0.03, p=0.005, p=0.04, respectively) (Figure 1A). At 4 weeks, the 10⁷ HUCBC group also became significantly less hyperactive than the stroke-only rats (p=0.003) and the groups 10⁴ and 3 to 5×10⁷ remained significantly less active (p=0.008 and p=0.02, respectively). Similarly, on horizontal activity, there was a significant reduction of movement in the 10⁴, 10⁵, and 3 to 5×10⁷ groups compared with the nonrecipient MCAO rats at 2 weeks (p=0.005 for all 3 groups), and at 4 weeks the 10⁶ and 10⁷ dose groups were significantly less active than the MCAO-only (both p=0.05) (Figure 1B). On stereotypy number and stereotypy time, there was no significant reduction in activity between media-treated and the HUCB-treated groups.

On clockwise rotation, total distance, and vertical activity, there were no interactions between HUCBC dose, time postinfusion, and observational period (ie, 12 test time points) (see right column of Table 1).
Elevated Body Swing Test
Before MCAO, all rats displayed relatively little bias (elevated body swing test) (p<0.05; #p<0.0001).

Step Test
Before MCAO, all rats took the same number of steps with the right and the left paw. Rats that had stroke but received only media infusion displayed an increase in asymmetry (expressed as percent of baseline difference between right and left steps) of ∼250% and 200% at 2 and 4 weeks, respectively (Figure 2B). Rats infused with the highest doses of HUCBC (10⁶ and 3 to 5×10⁷) showed significantly less asymmetry than the media-treated group. At 2 and 4 weeks, both these groups were performing at prestroke baseline levels.

HUCBC Delivery Reduces the Histological Damage Induced by MCAO
Although in the MCAO-only animals the striatum and cortex presented with a vast tissue loss, the administration of HUCBC 24 hours after MCAO significantly reduced the extent of ischemic brain damage, with the cortex and striatum remaining largely intact (Figure 3A). Infarct volume in the stroke group receiving media only (n=4) was 33.15±4.29%, whereas animals of the 10⁷ group (n=5) showed infarcts involving only 11.46±4.13% of the hemisphere (Figure 3B).

Delivered Cells Were Localized to Spleen and Brain as Determined by PCR and Immunohistochemistry
Human nuclei immunoreactive cells were detected only in the spleen and ipsilateral brain hemisphere of animals injected with 10⁶, 10⁷, and 3 to 5×10⁷ cell doses (Figure 4A). Here, the immunopositive cells were localized predominately to blood vessels, with few cells being detected in the parenchyma of either spleen or brain. To confirm the organ distribution of HUCBC at 4 weeks after MCAO, PCR analysis using HG3PDH was performed. We found HG3PDH...
in spleen and ipsilateral brain hemisphere (injured) of animals infused with high doses ($\geq 10^6$) of HUCBC (Figure 4B). However, in the lower HUCBC dose groups ($10^4$ and $10^5$), no HG3PDH was found (data not shown). Similarly, no HG3PDH was detected in the thymus, liver, bone marrow, kidney, or lung at any HUCBC dose (Figure 4B; kidney and bone marrow not shown).

**Discussion**

The results of the present study suggest that mitigation of MCAO-induced deficits depend on the number of HUCBC injected. Specifically, at 4 weeks after HUCBC infusion, the majority of the behavioral measures demonstrated that when $10^6$ or more HUCBC were used after permanent MCAO, there was significant recovery in behavioral performance; however, at lower doses ($10^4$ and $10^5$), although there was a similar tendency, this did not reach significance. Interestingly, at the highest HUCBC doses used (3 to $5 \times 10^5$), there was no further behavioral recovery. Curiously, the $10^7$ dose group in the elevated body swing test did not demonstrate any statistical improvement as shown by the spontaneous activity and step test for this group. However, review of individual performance on the elevated body swing test suggested the presence of 2 outliers ($>2.5$ SD from mean); when these were excluded from the analysis, there was statistically significant improvement on this test, as well ($p<0.031$).

In addition to behavioral improvements, this is the first report, to our knowledge, in which mononuclear cord blood cells are shown to reduce the ischemic volume, especially at $10^7$ HUCB or more cells. A number of studies using the rat MCAO model of stroke have previously described a lack in correlation between improved behavioral test performance and ischemic damage. In our study, the mismatch between behavioral and pathological measures observed in doses of HUCBC that are $<10^7$ suggest that these cells are mediating dose-dependent protective mechanisms that are manifest as behavioral improvements at doses equal to $10^6$ or as behavioral and brain pathologic recovery at doses $>10^6$.

Intravenously delivered HUCBC found in the brain 4 weeks after MCAO were exclusively localized to the ischemic hemisphere. Moreover, immunofluorescent localization of the HUCBC by human nuclei detection suggests that their numbers were small and mostly limited to the cerebrovasculature. The most cogent explanation is that the intravenously injected HUCBC may be following homing signals that attract them to the injured site. In fact, in vitro studies have shown that HUCBC can follow chemotactic cues from brain homogenates of stroke rats, and a number of in vivo studies have described tropism properties by intravenously delivered cells.

Besides the ipsilateral hemisphere, we also found HUCBC in the spleen. The function of the spleen as a secondary immune organ suggests it may be a tropic target for HUCBC and as such provides a putative mechanism by which HUCBC might modulate the immune system. Only $\approx 1\%$ of HUCBC is CD34-positive, the putative stem cell population; the major fractions are represented by immature lymphocytes ($\approx 65\%$) and monocytes ($\approx 30\%$). Interestingly, spleen-derived tolerogenic lymphocytes targeting activated blood vessel segments were shown to attenuate brain damage in rats subjected to MCAO, suggesting that in our study HUCBC infusion could be mediating functional recovery from MCAO at the level of the cerebrovasculature. Further, cord blood mononuclear cells have also been reported to express angio-
genic factors such as vascular endothelial growth factor and angiopoietin-1 and 2.22 After an ischemic insult, a cascade of inflammatory molecular and cellular events takes place and clinical studies have suggested that this acute response affects not only clinical outcomes but also the extent of brain injury.23 The rat MCAO model recapitulates many of the cellular parameters of brain inflammation seen in stroke. Therefore, HUCBC treatment may influence the cascade of inflammatory/immune events and thereby explain the neurobehavioral and histological benefits observed in this study. The mononuclear fraction of cord blood cells produces large amounts of IL-10,24 a potent anti-inflammatory cytokine, which may be involved in reducing the poststroke inflammatory response.

Molecular-based mechanisms may include not only immune processes mediated by interleukins but also the involvement of growth/ trophic factors. The mononuclear cord blood cells have been shown to express growth factors, such as nerve growth factor,25 and some investigators have hypothesized the major role of endogenous neuroprotective factors in the HUCB-induced ischemic brain recovery.26

Regardless of the mechanism of action, the clinical relevance of this HUCB-based therapeutic option for ischemic stroke is obvious, although additional work is needed before use of HUCBC in the clinical setting. For instance, one major obstacle will be attaining the number of cells needed for clinical benefit in man; our highest rat dose would translate into using the cells derived from >20 cords for just I dose of 107. In vitro expansion of the cells before infusion could resolve this issue.

Acknowledgments

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References

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