Delayed Administration of Human Umbilical Tissue-Derived Cells Improved Neurological Functional Recovery in a Rodent Model of Focal Ischemia

Li Zhang, MD; Yi Li, MD; Chunling Zhang, MD; Michael Chopp, PhD; Anna Gosiewska, PhD; Klaudyne Hong, PhD

Background and Purpose—The short time window required by neuroprotective strategies for successful treatment of patients with ischemic stroke precludes treatment for most. However, clinical therapies based on neuroregeneration might extend this therapeutic time window and thus address a significant unmet need. Human umbilical tissue-derived cells have shown great potential as neuroregenerative candidates for stroke treatment.

Methods—The effectiveness of intravenous administration of human umbilical tissue-derived cells was tested in a rodent middle cerebral artery stroke model in a dose escalation study (doses tested: \(3 \times 10^5\), \(1 \times 10^6\), \(3 \times 10^6\), or \(1 \times 10^7\) cells/injection) followed by a time-of-administration study (time after stroke: Day 1, Day 7, Day 30, and Day 90 at a dose of \(5 \times 10^6\) cells/injection). Controls were phosphate-buffered saline injections and human bone marrow-derived mesenchymal stromal cell injections. Post-treatment outcome tools included the modified neurological severity score and the adhesive removal tests. Histology was performed on all cases to evaluate synaptogenesis, neurogenesis, angiogenesis, and cell apoptosis.

Results—Statistically significant improvements of human umbilical tissue-derived cell treatment versus phosphate-buffered saline in modified neurological severity scores and adhesive test results were observed for doses \(\geq 3 \times 10^6\) cells up to 30 days poststroke. At doses \(\geq 3 \times 10^6\), histological evaluations confirmed enhanced synaptogenesis, vessel density, and reduced apoptosis in the ischemic boundary zone and increased proliferation of progenitor cells in the subventricular zone of human umbilical tissue-derived cell-treated animals versus phosphate-buffered saline controls.

Conclusions—These results indicate effectiveness of intravenous administration of human umbilical tissue-derived cells in a rodent stroke model compared with phosphate-buffered saline control and warrant further investigation for possible use in humans. (Stroke. 2011;42:1437-1444.)

Key Words: cerebral infarct ■ human umbilical cord ■ stroke recovery ■ synaptogenesis

Stroke is a leading cause of death and disability worldwide. Unfortunately, treatment of ischemic stroke remains one of the most challenging areas in medicine today. Over the last 20 years, cell-based therapies have emerged as a potential approach in the treatment of stroke.1–3 Transplantation of stem cells derived from fetal tissues as well as progenitor cells from bone marrow and human umbilical cord blood promoted neuronal survival, tissue repair, and, most importantly, recovery after experimental stroke.3–4 However, limited stem and progenitor cell sources along with ethical and safety concerns associated with the use of embryonic stem cells have spurred significant interest in the search for alternative stem cell sources for stroke treatment.

Human umbilical cord tissue contains stem cells and is a candidate source of cells for restorative therapy in stroke.7,8 Previous studies indicated that human umbilical tissue-derived cells (hUTC) secrete several neurotrophic factors such as brain-derived neurotrophic factor, fibroblast growth factor 2, and interleukin 6, all of which have the capacity to govern neurorestoration.9–12 However, the efficacy of systemically-administered hUTC to restore brain function in rodent stroke models has not been evaluated. In the present study, we tested whether intravenous administration of hUTC improves neurological functional recovery in rats after stroke. The effect of hUTC was compared with that of human bone marrow-derived mesenchymal stromal cells (hMSCs), which, when administered intravenously, have been shown to improve functional outcomes in rats after stroke.13,14

Methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. All
outcome measurements were performed by observers blinded to the treatments.

Animal Model
Male Wistar rats (n = 8 per cohort) weighing 270 to 300 g were subjected to 2 hours transient focal ischemia. Briefly, rats were anesthetized with 3.5% isoflurane and maintained with 1.0% to 2.0% isoflurane in 70% N2O and 30% O2 using a face mask. Rectal temperature was maintained at 37°C throughout the surgical procedure using a feedback-regulated water heating system. The right common carotid artery, external carotid artery, and internal carotid artery were exposed. A 4-0 monofilament nylon suture (18.5 to 19.5 mm), determined by the animal weight, with its tip rounded by heating near a flame, was advanced from the external carotid artery to the lumen of the internal carotid artery until it blocked the origin of the middle cerebral artery (MCA). Two hours after MCA occlusion, animals were reanesthetized with isoflurane and reperfusion was achieved by withdrawal of the suture until the tip cleared the lumen of the internal carotid artery.

Isolation and Preparation of hUTC and hMSCs
Human umbilical tissue-derived cells were isolated and cultured as described elsewhere. These cells are derived from the umbilical cord of a single donor and do not exhibit key hematopoietic markers such as CD34. In addition, these cells express HLA-ABC but do not express HLA-DR, DP, DQ, and do not express markers for antigen-presenting cells CD80 and CD86. Briefly, human umbilical cords were obtained with donor consent after live births from the National Disease Research Interchange (Philadelphia, PA). Tissues were minced and enzymatically digested in a cocktail of 50 U/mL collagenase containing 500 U/mL Dispase and 5 U/mL hyaluronidase (Sigma, St Louis, MO). After almost complete digestion, the cell suspension was passed through a 70-μm filter and centrifuged at 350 g. The cell pellets were washed in Dulbecco minimal essential medium and seeded at 5000 cells/cm² in 7-tissue culture (Corning, Corning, NY) in Dulbecco minimal essential medium–high glucose, 15% fetal bovine serum (HyClone, Logan, UT), β-mercaptoethanol (Sigma) + 100 μL/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Cells were expanded in culture under standard conditions in atmospheric oxygen with 5% carbon dioxide at 37°C. When cells reached approximately 70% confluence, they were passaged using trypsin/ethylenediaminetetraacetic acid (Gibco, Grand Island, NY) every 3 to 4 days and reseeded at a density of 5000 cells/cm² on gelatin-coated flasks. Cells were harvested after 10 passages (approximately 20 population doublings) and cryopreserved in growth medium containing 10% (vol/vol) dimethyl sulfoxide (Sigma) in a programmable rate freezer (Thermo Forma, Marietta, OH). Cells were stored in a unit maintaining a phase of nitrogen vapor with a small amount of liquid nitrogen at approximately −120°C until thawing for cell characterization or for administration in the study. Cell viability was reconfirmed on thawing and only samples meeting viability requirements were injected into animals. Thawed cell samples were immunophenotyped before use to ensure similar immunophenotypes as reported elsewhere, and cells were characterized to ensure safety and identity. Identity was evaluated by flow cytometry for the expression of several cell surface markers. Antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). Antibodies used in the analysis were IgG1-FITC, IgG-PE FITC, CD10, CD13, CD31, CD34, CD44, CD45, CD73, CD117, CD141, HLA-DR, DP, DQ, HLA-ABC, and PDGF-Rα.

Cell karyotype analysis was also performed to confirm that the cells contained the expected complement of human chromosomes. Cell pellets were evaluated by polymerase chain reaction for the presence of viral pathogens: HIV-1, HIV-2, cytomegalovirus, hepatitis B virus, hepatitis C virus, human T-lymphocytic virus, and Epstein-Barr virus.

Cells were prepared for injection into the MCA-injured animals immediately before delivery. Cell vials were thawed in a 37°C water bath and then washed with phosphate-buffered saline (PBS). Aliquots of the cell suspension were removed and mixed with Trypan blue and counted with a hemacytometer. The cell concentration in the cell suspension was adjusted to the appropriate density with PBS (3 × 10⁵, 1 × 10⁶, 3 × 10⁶, 5 × 10⁶, or 1 × 10⁷ in 2 mL PBS) for infusion.

Human mesenchymal stem cells were cultured according to instructions provided by the supplier (Cambrex Corp, East Rutherford, NJ).

Experimental Protocol
Rats subjected to MCA occlusion were randomly allocated to the following groups.

Dose Response
Six groups of 8 animals each were included in the dose–response study. All treatments were performed intravenously in 2 mL PBS, 24 hours after MCA occlusion: Group 1, hUTC at 3 × 10⁶ cells per injection; Group 2, hUTC at 1 × 10⁶ cells per injection; Group 3, hUTC at 3 × 10⁵ cells per injection; Group 4, hUTC at 1 × 10⁵ cells per injection; Group 5, 2 mL of PBS control only; and Group 6, hMSC at 3 × 10⁵ cells per injection. For identification of cell proliferation, rats received daily intraperitoneal injections of bromodeoxyuridine (BrDU, 100 mg/kg; Sigma), a thymidine analog, starting at 24 hours after stroke and subsequently for 14 consecutive days. All animals were euthanized at Day 60 poststroke.

Timing of Administration
Five groups of 8 animals each were included in this study based on the time of administration. Ideally, a PBS control group would have been needed for every time point evaluated. However, to limit as much as possible the number of animals euthanized in this study, the following experimental design was selected: Group 7, 5 × 10⁶ hUTC cells administered on Day 1 poststroke; Group 8, 5 × 10⁶ hUTC cells administered on Day 7 poststroke; Group 9, 5 × 10⁶ hUTC cells administered on Day 30 poststroke; Group 10, 5 × 10⁶ hUTC cells administered on Day 90 poststroke; and Group 11, 2 mL of PBS only administered on Day 30 poststroke, which served as a control group.

Functional Outcome
Modified Neurological Severity Score
The modified neurological severity score is a composite of motor, sensory, reflex, and balance tests. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). Animals were tested weekly for up to 60 days and biweekly thereafter.

Adhesive Removal Test
An adhesive removal test was used to measure somatosensory deficits. The mean time (seconds) required to remove stimuli from the left limb was recorded. Animals were tested 1 day before MCA occlusion and weekly thereafter starting 1 day after stroke onset. To increase the sensitivity of the test, rats were challenged with half-sized stimuli starting 70 days after stroke onset. The half-sized stimuli tests were conducted biweekly thereafter.

Measurements of Infarct Volume
Rats were euthanized 60 (dose–response study) or 140 days (timing of administration study) after stroke. Infarct volume was measured on 7 hematoxylin and eosin-stained coronal sections using a Global Laboratory Image analysis program (Data Translation, Marlboro, MA), as previously described. The infarct volume is presented as a percentage of total contralateral hemisphere volume.

Immunohistochemistry
Immunohistochemical studies were performed on coronal sections corresponding to bregma −1.0 to 1.0 mm obtained from each experimental animal. To identify proliferating cells, monoclonal antibody against BrdU (Dako) was used at a titer of 1:100. Double stainings were performed to visualize cellular colocalization of BrdU with neuron-specific marker NeuN (Chemicon) at a dilution of 1:500 and with glial fibrillary acidic protein (Dako), an astrocyte marker at a dilution of 1:10 000. For a morphological analysis of vessels, a...
polyclonal antibody against Von Willebrand factor (vWF; Dako) was used at a titer of 1:400. To detect presynaptic plasticity and synaptogenesis, a monoclonal antibody against synaptophysin (Chemicon) was used at a titer of 1:500. To detect neuroblasts, a polyclonal antibody against doublecortin (DCX; Santa Cruz) was used at a titer of 1:200. For single labeling of BrdU, vWF, synaptophysin, HM, and DCX, adjacent sections were incubated with the primary antibodies at 4°C. Sections were sequentially incubated with the appropriate biotinylated secondary antibodies (goat antimouse IgG for BrdU, synaptophysin, and HM; goat antirabbit IgG for vWF; and horse antigoat IgG for DCX). The signal was detected by avidin–biotin complex (Vector Laboratories) before staining with diaminobenzidine and hydrogen peroxide. Double immunofluorescent labeling of BrdU with NeuN and BrdU with glial fibrillary acidic protein were done in sections that were incubated with secondary antibodies conjugated with fluorescein isothiocyanate (Calbiochem) or cyanine (Jackson Immunoresearch). The terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling method (in situ apoptosis Detection Kit; Chemicon) was used to assess apoptotic cells according to the procedures provided by the manufacturer.

The adult subventricular zone (SVZ) contains self-renewing multipotent neural progenitors, which are capable of generating all 3 neuronal lineages.20–22 To examine whether administration of hUTC enhanced endogenous neurogenesis, proliferated SVZ cells identified by BrdU-positive cells and neuroblasts detected by DCX-immunoreactive cells were measured at 60 days after stroke onset. The numbers of BrdU-immunoreactive cells within the ipsilateral lateral ventricle wall were counted from each rat and divided by the ipsilateral lateral ventricle wall area to determine the cell density. The DCX-immunoreactive area within the ipsilateral lateral ventricle wall was measured and presented as percentage of area.

Synaptic plasticity in the ischemic penumbra region contributes to functional recovery after stroke.23 To examine whether administration of hUTC enhanced synaptogenesis, synaptophysin immunoreactivity was measured 60 days after stroke onset. Specifically, 8 fields of view from the ischemic boundary zone were digitized using a 40× objective from a MicroComputer Imaging Device (Imaging Research, St Catharines, Canada) system. The numbers of vWF-immunoreactive vessels and the positive area of synaptophysin in the ischemic boundary zone were measured throughout each field of view. Data are presented as density of vWF-immunoreactive vessels and percentage of synaptophysin-immunoreactive areas, respectively. To quantify apoptotic cells and human cells, numbers of terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling- and HM-positive cells throughout the ipsilateral hemisphere were counted. Data are presented as the total number of ipsilateral terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling- and HM-positive cells per section.

### Statistical Analyses

Behavioral data were evaluated for normality and the nonparametric analysis was considered if data did not follow a normal distribution. A 1-way analysis of variance was conducted to test the treatment effect on each outcome between the control and all dose groups at each time point. Histology data were evaluated using Student’s t test. A treatment effect was detected if probability value was <0.05.

### Results

A total of 97 animals underwent MCA occlusion, of which 9 died before completion of the study and were therefore excluded from the analyses. Of these 9 animals, 6 died immediately poststroke and were not subjected to treatment. Two animals died at Day 8 poststroke from Group 2–dose–response study (treated with hUTC at a dose of 1×10⁶ cells/injection) and 1 at Day 38 post stroke from Group 6–dose–response study (treated with hMSC at a dose of 3×10⁶ cells/injections). All animals that died during the study were replaced such that the final count of animals per group was 8.

### Neurological Functional Recovery

The dose–response study indicated that treatment with hUTC at doses of 3×10⁶ and 1×10⁷ cells/injection, but not at doses of 3×10⁵ and 1×10⁶ cells/injection, significantly improved the performance on adhesive removal test and modified neurological severity score compared with the PBS-treated rats starting at Day 14. This statistically significant difference persisted up to Day 60 poststroke. Treatment with hMSC exhibited a similar effect on neurological functional improvement as compared with hUTC at doses of 3×10⁶ and 1×10⁷ cells/injection (Figure 1).

The time of administration study indicated that hUTC administered 1 day after stroke onset significantly improved neurological function as measured by adhesive removal test and modified neurological severity score as compared with PBS treatment. This statistical significance was first seen at Day 7 and was maintained up to day 140 poststroke. Administration of hUTC at Day 7 or 30 significantly improved the performance on modified neurological severity score starting at Days 28 and 70, respectively, as compared with treatment with PBS. This statistical difference was maintained up to Day 140 poststroke. In addition, significant improvements on adhesive removal test were detected in rats treated with hUTC at Day 7 or 30, starting from Day 49 through 140, compared with PBS-treated rats. Administration of hUTC on Day 90 poststroke significantly improved performance on the adhesive removal test as observed on Day 140 but failed to improve the modified neurological severity score score compared with PBS-treated rats. Our data thus indicate that delayed administration of hUTC up to 30 days improved neurological functional recovery (Figure 1).

### Infarct Volume

No significant reduction of infarct volume was detected in rats treated with hUTC regardless of dose and time of administration when compared with rats treated with PBS. In addition, treatment with hMSC did not significantly reduce the lesion volume compared with PBS-treated rats (Table).

### Detection of Human Cells

Few HM-immunoreactive cells were detected within the ipsilateral hemisphere of rats treated either with hUTC or hMSC. Specifically, HM-positive cells ranged from 1.4±1.6 cells/section in hUTC-treated animals. The number of HM-immunoreactive cells did not differ between the experimental groups. No HM-immunoreactive human cells were found in PBS-treated rats 90 days after stroke.

### SVZ Cell Proliferation

Treatment with hMSC and with hUTC at doses of 3×10⁶ and 1×10⁷ cells/injection but not at doses of 3×10⁵ and 1×10⁶ cells/injection significantly increased the number of ipsilat-
eral BrdU-immunoreactive cells in the SVZ compared with PBS-treated rats. Treatment with hUTC thus dose-dependently increased SVZ cell proliferation in rats after stroke (Figure 2A–B). In addition, treatment with hUTC at a dose of $3 \times 10^6$ cells/injection significantly increased the DCX-immunoreactive area in the ipsilateral SVZ (Figure 2C–D) compared with that of PBS-treated rats when the treatment was initiated at 24 hours after stroke onset, indicating that hUTC treatment enhanced neuroblast concentration in the ischemic brain.

Double-staining immunohistochemistry revealed that fewer than 5% of BrdU-positive cells were reactive for NeuN and glial fibrillary acidic protein across groups. Treatment with hUTC at doses of $3 \times 10^6$ did not significantly increase the proportion of BrdU/NeuN (2.7% ± 0.5% versus 2.1% ± 0.5%) or BrdU/glial fibrillary acidic protein (3.3% ± 0.5% versus 4.1% ± 0.8%) double-labeled cells compared with PBS-treated rats 60 days after stroke onset.

### Table. Treatments and Lesion Volumes for All Groups

<table>
<thead>
<tr>
<th>Groups (n=8/Group)</th>
<th>Treatments</th>
<th>Lesion Volume (% of Contralateral Hemisphere)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>hUTC at $5 \times 10^5$ at Day 1 after MCA</td>
<td>34.9 ± 2.2</td>
</tr>
<tr>
<td>Group 2</td>
<td>hUTC at $1 \times 10^6$ at Day 1 after MCA</td>
<td>34.3 ± 2.3</td>
</tr>
<tr>
<td>Group 3</td>
<td>hUTC at $5 \times 10^6$ at Day 1 after MCA</td>
<td>33.2 ± 1.9</td>
</tr>
<tr>
<td>Group 4</td>
<td>hUTC at $1 \times 10^7$ at Day 1 after MCA</td>
<td>33.8 ± 2.1</td>
</tr>
<tr>
<td>Group 5</td>
<td>PBS at Day 1 after MCA</td>
<td>34.9 ± 2.6</td>
</tr>
<tr>
<td>Group 6</td>
<td>hMSC at $5 \times 10^6$ at Day 1 after MCA</td>
<td>34.6 ± 2.2</td>
</tr>
<tr>
<td>Group 7</td>
<td>hUTC at $5 \times 10^6$ at Day 1 after MCA</td>
<td>31.3 ± 2.6</td>
</tr>
<tr>
<td>Group 8</td>
<td>hUTC at $5 \times 10^6$ at Day 7 after MCA</td>
<td>33.0 ± 2.4</td>
</tr>
<tr>
<td>Group 9</td>
<td>hUTC at $5 \times 10^6$ at Day 30 after MCA</td>
<td>31.3 ± 2.1</td>
</tr>
<tr>
<td>Group 10</td>
<td>hUTC at $5 \times 10^6$ at Day 90 after MCA</td>
<td>34.0 ± 2.5</td>
</tr>
<tr>
<td>Group 11</td>
<td>PBS at Day 30 after MCA</td>
<td>33.9 ± 2.4</td>
</tr>
</tbody>
</table>

hUTC indicates human umbilical tissue-derived cells; MCA, middle cerebral artery; hMSC, human bone marrow-derived mesenchymal stromal cells; PBS, phosphate-buffered saline.

Figure 1. Adhesive removal test and modified neurological severity score for all groups up to 60 days poststroke for the dose–response study and 140 days poststroke for the time-of-administration study. All animals in the dose–response study were treated on Day 1 at doses indicated on the graph. Animals in the hUTC groups of the time-of-administration study were treated with $5 \times 10^6$ cells/injection at times indicated on the graph. Before the stroke, there was no significant difference in neurological function measured by adhesive removal test, as indicated on A, C, and modified neurological severity scores for normal, preischemic rats were equal to 0.0 for all animals (not shown on graphs). A, Adhesive removal test of rats in the dose–response study; (B) modified neurological severity score of rats in the dose–response study; (C) adhesive removal test of rats in the time-of-administration study; and (D) modified neurological severity score of rats in the time-of-administration study. *P<0.05 as compared with the phosphate-buffered saline-treated group. hUTC indicates human umbilical tissue-derived cells.

Figure 2. A, Representative micrographs showing BrdU immunoreactivity at the ipsilateral SVZ acquired from rats treated with PBS (top panel) and hUTC at a dose of $3 \times 10^6$ cells (bottom panel); (B) quantitative analysis of BrdU-positive cell density (number of cells/mm²) in the ipsilateral and contralateral ventricle walls; (C) representative micrographs of DCX immunoreactivity at ipsilateral hemispheres from rats treated with PBS (left panel) and hUTC at a dose of $3 \times 10^6$ cells (right panel); (D) quantitative analysis of DCX expression in the SVZ. Bars = 50 μm. *P<0.05 as compared with the PBS-treated group. BrdU indicates bromodeoxyuridine; SVZ, subventricular zone; PBS, phosphate-buffered saline; hUTC, human umbilical tissue-derived cells; DCX, doublecortin.
Synaptogenesis
Administration of hUTC at doses of $3 \times 10^6$ and $1 \times 10^7$ cells/injection, but not at the lower doses of $3 \times 10^5$ and $1 \times 10^6$ cells/injection, as well as treatment with hMSC significantly increased synaptophysin expression in the ischemic boundary area compared with that observed in animals treated with PBS (Figure 3).

Vascular Density at the Ischemic Boundary Zone
Treatment with hUTC at doses of $3 \times 10^6$ and $1 \times 10^7$ cells/injection, but not at the lower doses of $3 \times 10^5$ and $1 \times 10^6$ cells/injection, as well as treatment with hMSC significantly increased vWF-immunoreactive vascular density in the ischemic boundary area compared with that observed in PBS-treated rats at 60 days after stroke onset (Figure 4).

Apoptotic Cell Death
Scattered terminal deoxynucleotidyl transferase–mediated dUTP-biotinic end labeling-positive cells were present throughout the ipsilateral hemisphere. These cells were primarily located in the ischemic boundary area 60 days after stroke onset. Treatment with hUTC at doses of $3 \times 10^6$ and $1 \times 10^7$, but not at the lower doses of $3 \times 10^5$ and $1 \times 10^6$, as well as treatment with hMSC significantly reduced the number of terminal deoxynucleotidyl transferase–mediated dUTP-biotinic end labeling-positive cells compared with that observed in PBS-treated rats. Treatment with hUTC thus reduced apoptotic cell death in a dose-dependent manner (Figure 5).

Discussion
Intravenous administration of hUTC in a rodent MCA model 24 hours after stroke dose-dependently promoted neurological functional recovery, which persisted at least 2 months after stroke onset. Significant functional recovery was also observed when treatment was initiated at Day 7 or 30 but not at Day 90 after stroke onset. Treatment with hUTC enhanced SVZ cell proliferation, synaptogenesis, and vessel density and reduced apoptotic cell death in the ischemic boundary area, which likely contributed to the improvement of neurological functional recovery in rats after stroke.

Prior research indicates that intracerebral implantation of human umbilical cord-derived mesenchymal stem cells at 2 weeks after stroke onset reduces ischemic brain damage and neurological functional deficits in rats, indicating a neuroprotective effect of these cells in experimental stroke.7 In the present study, intravenous administration of hUTC improved neurological function recovery without the reduction of lesion volume compared with PBS-treated rats, thus suggesting that the improvement of neurological function observed here was derived from neurorestorative effects in the ischemic boundary area, resulting in neurogenesis, synaptogenesis, and angiogenesis. These regenerative processes have previously been shown to contribute to the functional recovery after ischemic insult.24–27 Neurogenesis is initiated with neural progenitor cell proliferation after stroke.28 Treatment with hUTC significantly increased the number of BrdU-incorporating cells and the DCX-immunoreactive area in the ipsilateral SVZ suggesting that hUTC treatment enhanced
endogenous neurogenesis. Neuronal differentiation was further evaluated here by the presence, albeit in small number, of BrdU-immunoreactive cells that also coexpressed NeuN, a neuronal marker. In our study, there was no difference in BrdU–NeuN coexpression results between hUTC- versus PBS-treated animals. This may be due to the fact that the BrdU pulse labeling methodology used here only allowed detection of cell proliferation within the first 2 weeks poststroke and thus did not detect neuronal differentiation at later stages. Based on these results, and despite the positive DCX results, the effect of hUTC on neuronal differentiation remains to be determined. Therefore, the data associating neurogenesis with functional recovery should be interpreted with caution. In the present study, treatment with hUTC increased synaptophysin immunoreactivity at the ischemic boundary area, suggesting that hUTC treatment enhanced synaptogenesis. As for angiogenesis, a physiological repair process that naturally occurs in the ischemic brain,29 the increased vWF-immunoreactive vessel density in the rats treated with hUTC indicated that hUTC modulated the vascular system. Collectively, our data therefore indicated that intravenous administration of hUTC promoted endogenous neural progenitor proliferation, synaptogenesis, and increased vessel density, which may explain how hUTC also improved functional outcomes in the rats. Although multiple prior studies have reported that implantation of exogenous stem/progenitor cells from multiple sources can enhance neurogenesis, synaptogenesis, and angiogenesis,13,25,30–32 our study is the first to suggest a similar outcome after intravenous administration of hUTC.

Apoptotic cell death has been identified in normal brain throughout life and plays a critical role in maintaining tissue homeostasis.33,34 Apoptosis is a common feature in neurogenic regions during development and in the adult brain, in which the steady production of newly generated cells is largely eliminated through an apoptotic mechanism.35 We have previously demonstrated that stroke-induced apoptotic cell death peaked at 1 to 2 days poststroke and persisted for at least 4 weeks after stroke onset.35 In the present study, hUTC treatment was associated with reduced apoptotic cell death at 2 months after stroke onset compared with controls, suggesting that hUTC treatment not only enhances SVZ cell proliferation, but also reduces the apoptotic cell death in the damaged brain tissue. Prior research has shown that, although endogenous neurogenesis occurs in response to ischemic injury, the majority (approximately 80%) of newly generated cells die after stroke, indicating that apoptosis contributes significantly to the fate of newly generated cells.31,36 Thus, it is reasonable to speculate that by reducing apoptotic cell death, hUTC treatment may improve the survival of newly generated cells, an effect that might be linked to the functional recovery observed in the animals after the stroke. The effects of intravenous hUTC treatment on the survival of newly generated cells after stroke warrant further investigation.

Previous studies have indicated that hMSCs are multipotent cells that, when implanted directly into the ischemic rat brain, can integrate into the parenchymal brain tissue and express neuronal markers 3 weeks after intracerebral transplantation.7,37 However, in the present study and after intravenous administration of hUTC, few human cells were detected in the ischemic brain 2 months after treatment, suggesting that cells were eliminated from the host brain over time.

In support of this hypothesis is the fact that hUTC were previously shown to elicit a host tissue immune response, especially under inflammatory conditions.38 Stroke triggers an inflammatory response, which contributes significantly to the progression of brain damage.39 Thus, it is possible that transplanted cells were scavenged by the host immune system. In the hemiparkinsonian rats, the engrafted human umbilical cord matrix cells disappeared 6 weeks after transplantation, which is consistent with our finding.7,37 Therefore, although hMSCs have the potential to differentiate into parenchymal cells, the limited number of human cells observed in the present study suggests that neural transdifferentiation and fusion are unlikely the predominant mechanisms of action of intravenous administration of hUTC in stroke.

Considering that functional recovery was observed in our study, along with angiogenesis, synaptogenesis, and reduction of apoptotic cell death, it can be hypothesized that the mechanism of action of intravenous hUTC administration is linked to expression of neurotrophic factors from the cells. The hUTC secrete several neurotrophic factors such as brain-derived neurotrophic factor and fibroblast growth factor...
2, all of which have the capacity to induce neurogenesis, angiogenesis, and synaptogenesis after stroke.\(^{40,41}\) In addition, the antiapoptotic signaling pathway is activated by neurotrophic factors.\(^{42}\) Thus, although not investigated directly here, the enhancement of host brain plasticity and suppression of apoptosis observed in the present study are likely attributable to the paracrine neurotrophic action of hUTC. This hypothesis is supported by others who showed that administration of hMSCs increased neurotrophic factor expression, which in turn is known to augment host brain plasticity and reduce apoptotic cell death in the experimental stroke.\(^{14}\)

**Summary**

The intravenous administration of hUTC augmented endogenous neurorestorative responses and neurological functional recovery with a time of administration window up to 30 days after stroke onset in the rat MCA occlusion model. Intravenous administration of hUTC thus represents a novel treatment for further evaluation with a potential broad time of administration window after the treatment of stroke.

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**Disclosures**

K.H., Stroke Team Leader, and A.G., Senior Director of R&D, are both employees of ATRM.

**References**


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Abstract

Background and Objectives: To develop successful treatments for ischemic stroke patients, strategies are needed to extend the therapeutic window, as most treatments are currently limited by a short time window for treatment. However, cell-based regenerative treatments might provide an opportunity for longer therapeutic windows for treating previously untreatable issues. Umbilical cord tissue-derived cells have been shown to hold great promise as a therapy for stroke.

Methods: To evaluate the intravenous administration of umbilical cord tissue-derived cells in a rodent model of middle cerebral artery occlusion, we performed a dose-escalation study (doses: 3 × 10^5, 1 × 10^6, 3 × 10^6, or 1 × 10^7 cells per injection) and a subsequent administration time study (treatment times: Day 1, Day 7, Day 30, and Day 90). Controls included saline injections or injections of human bone marrow-derived mesenchymal stem cells (hMSCs).

Results: Treatment with 3 × 10^6 cells on Day 30 significantly improved neurological function and BrdU-positive cell counts compared to saline treatment. Microscopic analysis on Day 90 revealed a significant increase in synapse formation, vascular density, and reduction of apoptosis in the hUTC-treated group compared to controls that received saline.

Conclusion: These results demonstrate the efficacy of intravenous administration of umbilical cord tissue-derived cells in a rodent model of stroke, providing further evidence for the potential use of these cells in human stroke patients.