Human Neural Stem Cell Transplantation Promotes Functional Recovery in Rats With Experimental Intracerebral Hemorrhage

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Background and Purpose—Cell transplantation has been used to reduce behavioral deficit in cerebral ischemia. However, there is no report about cell transplantation in experimental intracerebral hemorrhage (ICH). We hypothesize that intravenously transplanted human neural stem cells (NSCs) can migrate and differentiate into neurons or glial cells, thereby improving functional outcome in ICH.

Methods—Experimental ICH was induced by intrastratial administration of bacterial collagenase in adult rats. One day after surgery, the rats were randomly divided into 2 groups to receive intravenously either immortalized Lac z–positive human NSCs (5×10⁶ cells in 500 μL, n=12) or the same amount of saline (n=13). The animals were evaluated for 8 weeks with modified limb placing and rotarod tests. Transplanted NSCs were detected by X-gal histochemistry or β-gal immunohistochemistry with double labeling of GFAP, NeuN, neurofilament, or CNPase.

Results—Intravenously transplanted NSCs migrated selectively to the perihematomal areas and differentiated into neurons (~10% of β-gal⁺ cells) and astrocytes (~75%). The NSC-transplanted group showed better functional performance on rotarod test after 2 weeks and on modified limb placing test after 5 weeks compared with the control group (P<0.05), and these effects persisted for up to 8 weeks. There was no difference in the final hemispheric area between the 2 groups.

Conclusions—Intravenously transplanted NSCs can enter the rat brain with ICH, survive, migrate, and improve functional recovery. Transplantation of human NSCs can be used to restore neurological deficits in experimental ICH. (Stroke. 2003;34:2258-2263.)

Key Words: intracerebral hemorrhage ■ stem cells ■ transplantation ■ rats

Intracerebral hemorrhage (ICH) represents at least 10% of all strokes in the Western population¹ and a considerably higher proportion in the oriental and black populations.² The prognosis of patients after ICH is poor, often much worse than that of patients with ischemic strokes of similar size. Patients with ischemic stroke (up to 30%) undergo hemorrhagic transformation,³ and a considerable number of ischemic infarct patients after thrombolysis develop hemorrhagic conversion or symptomatic hematoma.⁴ Currently, there is no available medical therapy for patients with ICH, and supportive care or invasive neurosurgical evacuation of hematoma in selective patients is all that can be done.

Neural transplantation has been used experimentally for Parkinson’s disease, epilepsy, and cerebral infarction.⁵,⁶ There are some reports of cell transplantation in ischemic infarct models. Various cells sources such as rodent mesenchymal stem cells,⁷-¹⁰ human bone marrow stem cells,¹¹,¹² human umbilical cord blood cells,¹³ rodent embryonic hippocampal formation cells,¹⁴ MHP36, a conditionally immortalized neuroepithelial cell line derived from embryonic mouse,¹⁵,¹⁶ and human teratocarcinoma-derived neurons¹⁷,¹⁸ ameliorated neurological deficits induced by experimental brain ischemia. Even in humans with ischemic infarct, some functional improvement has been reported with NT2/D cell line grafts, which are developed from human teratocarcinoma.¹⁹ However, to the best of our knowledge, no cell transplantation experiments in ICH models have yet been reported, and only one report of neural tissue graft did not show any functional improvement.²⁰

Neural stem cells (NSCs) are immature cells with the ability to renew themselves and give rise to neurons, astrocytes, and oligodendrocytes. These cells persist in the subventricular zone, hippocampus, cortex, and spinal cord, even in the adult.²¹ Isolated NSCs are able to proliferate in...
response to basic fibroblast growth factor or epidermal growth factor, and when the culture conditions are altered, they differentiate into several phenotypes of neurons. Furthermore, neurons derived from NSCs form functional synapses in vitro and in vivo.22 These results suggest that NSCs have the potential to differentiate into appropriate neurons to form a functional neuronal circuitry.

We tested whether human NSCs can survive, migrate, and differentiate into neurons or glial cells and whether they improve functional outcome in experimental ICH.

Materials and Methods

ICH Model
Twenty-five male Sprague-Dawley rats (Samtako, Osan) weighing 240 to 280 g were used in this study. All experimental procedures were approved by the Care of Experimental Animals Committee of the University Hospital and by the institutional review board for the use of human cells. ICH was induced by stereotaxic, intrastratal administration of bacterial collagenase by previously described methods.23 In brief, after an intraperitoneal injection of 1% ketamine (30 mg/kg) and xylazine hydrochloride (4 mg/kg), the rats were placed in a stereotaxic frame (Kopf Instruments). A burr hole was made, and a 30-gauge needle was inserted through the burr hole into the striatum (coordinates: 0.2 mm posterior, 6.0 mm ventral, and 3.0 mm lateral to the bregma). ICH was induced by the administration of collagenase type IV (1 μL saline containing 0.23 U, Sigma) over a period of 5 minutes. After placement for another 4 minutes, the needle was gently removed. The burr hole was sealed with bone wax, and the wound was sutured. Rectal temperature was maintained at 37 ± 0.5°C by use of a thermistor-controlled heat blanket. Animals were maintained in separate cages at room temperature, 25°C, with free access to food and water under a 12-hour light-dark cycle. After 3 weeks, the amount of pellets was restricted to 30 g/d to control weight.

Cell Preparation and Transplantation Procedure
Primary dissociated cell cultures were prepared from embryonic human brains of 15 weeks gestation as described previously.24–26 To provide an unambiguous molecular tag for identifying the implanted cells, the cell line was infected with a replication-incompetent retroviral vector encoding β-galactosidase (β-gal; Lac z) and puromycin-resistant genes. Dissociated cells prepared from human embryonic cerebrum were initially grown in serum-free medium supplemented with basic fibroblast growth factor. The cerebrum cultures were retrovirally transduced with the v-myc oncogene and subsequently cloned. One clone was called HB1.F3 and studied further. HB1.F3 cells were cultured in polylysine-coated culture dishes, which could be subcultured and passed weekly over a period of 6 months. HB1.F3 cells grown on coverslips were immunoreaction positive for nestin, the cell type-specific marker of NSCs. Detailed cell line characteristics are described in previous reports.24–26

Twenty-four hours after surgery, we performed behavioral tests and randomly selected rats (n = 12) received 500 μL suspension of HB1.F3 cells (5 × 10⁶ cells) slowly for 5 minutes via the tail vein. The same volume of saline was injected into the control group (n = 13). Immunosuppressants were not used in any of the animals.

Behavioral Testing
Behavioral testing was performed weekly with the rotarod and modified limb placing tests, which were monitored by 2 individuals blinded to rat treatment status. In the rotarod test,9 the rats were placed on the rotarod cylinder, and the time the animals remained on the rotarod was measured. The speed was slowly increased from 4 to 40 rpm within a period of 5 minutes. The trial was ended if the animal fell off the rungs or gripped the device and spun around for 2 consecutive revolutions. The animals were trained for 3 days before stereotactic operation. The maximum duration (in seconds) on the device was recorded with 3 rotarod measurements 1 day before ICH induction. Motor test data are presented as percentages of the maximal duration compared with the internal baseline control (before ICH).

The modified limb placing test is a version of a test previously described in the literature.27 The test consists of 2 limb-placing tasks that assess the sensorimotor integration of the forelimb and the hind limb by checking responses to tactile and proprioceptive stimulation. First, the rat is suspended 10 cm over a table, and the stretch of the forelimbs toward the table is observed and evaluated: normal stretch, 0 points; abnormal flexion, 1 point. Next, the rat is positioned on the edge of the table, with its forelimbs suspended over the edge and allowed to move freely. Each forelimb (forelimb, second task; hind limb, third task) is gently pulled down, and retrieval and placement are checked. Finally, the rat is placed toward the table edge to check for lateral placement of the forelimb. The 3 tasks are scored in the following manner: normal performance, 0 points; performance with a delay (2 seconds) and/or incomplete, 1 point; no performance, 2 points. A total of 7 points means maximal neurological deficit, and 0 points means normal performance. Additionally, the body weights of all animals were checked weekly for 8 weeks.

Histological Examination
At the end of behavioral testing, each animal was reanesthetized and perfused through the heart with 100 mL cold saline and 100 mL of 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline. After 24 hours of fixation in 4% paraformaldehyde, the brains were cryoprotected with 30% sucrose for 24 hours and cut by cryostat (Leica CM 1900) into 30-μm sections. Three sections through the needle entry site, which was identifiable on the brain surface, and sites 1.0 mm anterior and 1.0 mm posterior to plane were Nissl stained to analyze the hemisphere area. These sections are representative of the core of the hematoma. The total hemispheric areas of each section were traced and measured with an image analysis system (Image-Pro Plus, Media Cybernetics). The morphometric analyses involved computer-assisted hand delineation of the area of the striatum, cerebral cortex, and ventricle, as well as the whole hemisphere.

Adjacent serial coronal sections were processed for β-gal immunofluorescent staining with anti-β-gal antibody (1:200, Sigma) to identify the Lac z-positive, transplanted cells. A neurofilament (NF for neurons; 1:200; Sigma), neuronal nuclear antigen (NeuN for neurons; 1:200; Chemicon), glial fibrillary acidic protein (GFAP for astrocytes; 1:1000; Sigma), and 2′,3′ cyclic nucleotide 3′-phosphodiesterase (CNPase for oligodendrocytes, 1:300; Sigma) antibodies were used as cell type-specific markers. Primary antibodies were incubated overnight at 4°C with the free floating method, and both FITC-conjugated anti-mouse IgM and Cy3-conjugated anti-mouse IgG antibodies (1:300, Jackson Immunoresearch) were incubated for 1 hour at room temperature. Negative control sections from each animal were prepared for immunohistochemical staining in an identical manner except the primary antibodies were omitted. The colocalization of β-gal with neuronal and glial markers was performed by laser scanning confocal microscopy with a Bio-Rad MRC 1024 (argon and krypton). For immunofluorescence double-labeled coronal sections, green (FITC for β-gal) and red (Cy3 for NeuN, NF, CNPase, and GFAP) fluorochromes on the sections were excited by laser at 488 and 550 nm. Emissions were acquired sequentially with 2 separate photomultiplier tubes at 522 and 570 nm, respectively.

To determine graft survival semiquantitatively, transplanted cells in the same focal plane were counted bilaterally in 6 regions of interest (ROIs) situated within the striatum, cortex, and septal areas, and in each case, measurement was made in a predefined field (0.3 × 0.3 mm). The purpose of the standardized ROI cell counts was not to estimate the total number of grafted cells but to obtain an accurate count within selected ROIs to compare between both hemispheres.16 Two independent investigators counted the Lac z-positive cells (n = 12 for the NSC-transplanted group) and cells...
In the rotarod test (A), NSC-transplanted group showed better performance from 14 days on, and these benefits continued up to 8 weeks \((P<0.05)\). In the modified limb placing test (B), the ICH-only group showed slightly better performance at 7 and 14 days (5 weeks) on, the NSC-transplanted group showed better performance, and this continued up to 8 weeks \((P<0.05)\).

**Statistical Analysis**

All data in this study are presented as mean±SD. Data were analyzed by unpaired Student’s \(t\) test if they were normally distributed (Kolmogorov-Smirnov test, \(P>0.05\)). Otherwise, we used the Mann-Whitney \(U\) test and specified the test used. Two-tailed value of \(P<0.05\) was considered significant.

**Results**

**Human NSCs Improved Functional Deficits in Rats With Experimental ICH**

The NSC-transplanted group showed better performance on the rotarod test after 2 weeks compared with the control group (Figure 1A; \(P<0.05\)), and the effect persisted for up to 8 weeks. During the initial 2 weeks, the transplantation group showed slightly poorer performance on the limb placing test, but there was no statistically significant difference up to 4 weeks. From the fifth week, the NSC-transplanted group showed better results than the control group (Figure 1B; \(P<0.05\)). Initial body weights were similar (267.4±37.1 versus 258.9±35.7 g, \(P=0.56\)), and those after 8 weeks were not significantly different (314.0±36.6 versus 317.5±55.7 g, \(P=0.85\)). Additionally, the body weights of the 2 groups over the entire 8 week period were not different significantly (data not shown).

**Intravenously Transplanted Human NSCs Enter the Rat Brain, Survive, and Differentiate Into Astrocytes and Neurons**

\(\text{Lac}~\gamma\)-positive NSCs migrated selectively to the perihematomal areas and differentiated into neurons and astrocytes (Figures 2A and 3). The number of migrated \(\text{Lac}~\gamma\)-positive cells in 2 cortical ROIs and 3 striatal ROIs were 910.6±190.0 and 1037.6±239.2 cells/mm\(^2\), respectively (Figure 2B). The septal area showed 704±152.7 \(\text{Lac}~\gamma\)-positive cells/mm\(^2\). Transplanted cells were rarely found in the nonlesion, contralateral areas (cortex, 80.8±30.1/mm\(^2\); striatum, 93.7±38.9/mm\(^2\); septal area, 87.5±32.9/mm\(^2\)). Most (75%) of the transplanted \(\text{Lac}~\gamma\)-positive cells were also GFAP-positive and took the morphology of the endogenous astrocytes (Figures 2C and 3). Many GFAP\(^+\) \(\beta\)-gal\(^-\) cells were found along the needle tracks. A smaller portion (10%) became NeuN-positive neuronal cells in the lesonal hemisphere and \(\sim 4\%\) in the intact hemisphere (Figures 2D and 3), and NeuN\(^+\) \(\beta\)-gal\(^-\) cells were found in the vicinity of the hematoma and were intermingled with the GFAP\(^+\) \(\beta\)-gal\(^-\) astrocytes. No double labeling for the oligodendrocytic marker (CNPase) was observed.

**Human NSC Transplantation Did Not Reduce Hemispheric Atrophy After ICH**

Hemispheric area analysis showed a significant atrophy of lesion hemisphere (left side) in both the control group and the NSC-transplanted group (36.9±6.1 to 28.7±8.7 mm\(^2\) and 37.4±5.9 mm\(^2\) to 30.9±6.7 mm\(^2\); \(P<0.001\), respectively; Figure 2E). The areas of the striatum in both the ICH-only group and NSC-transplanted group were not different significantly (contralateral striatum, 14.8±3.5 versus 16.8±4.5 mm\(^2\), \(P=0.23\); ipsilateral striatum, 11.1±5.2 to 14.2±3.9 mm\(^2\), \(P=0.10\); Figure 2E). In this model of ICH, cortical atrophy was not different in either group (4.3±2.6% versus 4.4±2.0%, \(P=0.95\) by Mann-Whitney \(U\) test; Figure 2E and the Table). The human NSC-transplanted group showed lesser striatal atrophy compared with the control group, but it did not reach statistical difference (15.5±4.7% versus 25.0±8.0%, \(P=0.26\) by Mann-Whitney \(U\) test; the Table).

**Intravenously Administered Human NSCs Incorporate Some Systemic Organs But Do Not Show Tissue Destruction or Tumor Formation**

The liver and heart contained no \(\beta\)-gal\(^-\) positive cells. In the kidneys, lungs, and spleen, \(\beta\)-gal\(^-\) positive cells were found; however, no tissue damage such as leukocyte infiltration or tumor formation was detected in the histological examinations (hematoxylin and eosin staining). Also, there was no tumor growth in the brain.
Discussion

Our study shows that neural transplantation with human NSCs promotes functional recovery in the experimental ICH model after 2 weeks. Intravenously administered NSCs enter the rat brain, survive, and migrate to the perihematomal area. Most of the transplanted cells differentiate into glial cells (75%), and 10% of them differentiate into neurons. These findings reinforce that not only ischemic infarction but also ICH are good candidates for cell transplantation therapy. Systemically injected human NSCs are incorporated into some organs such as the kidneys, lungs, and spleen, and no tissue destruction or tumor formation was observed. Also, no abnormal tissue growth occurred in the brain.

Selective neuronal loss such as in the case of Parkinson’s disease has already been considered a good candidate for neural replacement therapy. ICH is associated with considerable mechanical disruption of tissue in a large portion of the brain, including the neural and glial cells, and it was generally accepted that this disorder would be less likely to benefit from neural transplantation. There has been only 1 report on the transplantation of 14-day-old fetal rat brain tissue into the site of the ICH after 12 days of ICH induction, which showed that the transplants were viable in only half of the recipients. The viable tissue transplant developed into mature glial tissue and ganglion cells but did not improve the behavioral deficits. Furthermore, fetal central nervous system tissue
transplantation has limitations of both practical (In Parkinson’s disease, 4 to 8 fetuses are required) and ethical problems. On the other hand, recent progress in the biology of NSCs has made it possible to routinely multiply NSCs obtained from a small amount fetal central nervous system tissue.

We used the HB1.F3 human NSC line, which was derived from the human embryonic brain (ventricular zone) at 15 weeks of gestation and was genetically engineered to have a marker gene, Lac-Z, with immortalization induced by means of v-myc insertion. After transplantation, v-myc expression was reported to be undetectable after 24 or 48 hours by endogenous signal. The loss of v-myc expression from stably engrafted NSCs after transplantation is consistent with the invariant absence of brain tumors derived from implanted v-myc–propagated NSCs in mice even after several years. As with mouse NSCs, neoplasms have never been observed with the human NSC line used in our study. Although rodent mesenchymal stem cell or human bone marrow stem cell transplantation showed functional improvement in the ischemic infarct model, recent reports indicated that bone marrow cells or hematopoietic stem cells could not differentiate spontaneously into neural cells in vivo, even in the case of injury to the nervous system, which evinced the conclusion that stem cells with hematopoietic lineage could differentiate into neural cell types in a very special experimental setting.

One of the mechanisms of functional improvement in neural transplantation is the replacement or augmentation of neural circuits by the transplanted NSCs; the other is associated with the release of trophic factors from the transplanted cells. Neurons derived from NSCs can form functional synapses in vitro and in vivo. However, transplanted NSCs can be functionally active cortical pyramidal neurons after 4 weeks by immunohistochemistry and patch-clamp techniques, and the early improvement on the rotarod test observed in our experiment 2 weeks after transplantation could not be explained solely by functional neural integration. The functional performance of the NSC-transplanted group on the limb placing test after 5 weeks was better than that of the control group, which might be related to neural integration. NSCs might induce synaptic plasticity in the host brain, for example, thus enabling the damaged brain to generate new connections. Recently, human bone marrow stromal cell transplantation in the rat focal ischemia model induced an increase in the amount of brain-derived neurotrophic factor and nerve growth factor 7 days after ischemia. Additionally, apoptotic cells significantly decreased in the ischemic boundary zone. Because few (1% to 5%) bone marrow stromal cells expressed the proteins phenotypic of brain parenchymal cells, the neurological benefits resulting from marrow stromal cell treatment may be derived from the increase in the amount of growth factors present in the ischemic tissue and the reduction of apoptosis in the penumbral zone. Measurements of the time course of neuronal differentiation and measurements of trophic factors likely would allow the separation between functional neural integration and trophic effects of NSCs.

We transplanted NSCs intravenously into rats on the day after ICH induction. However, a recent report of neural progenitor transplantation in the spinal cord contusion model showed that adult spinal cords are not absolutely noneuro-

| Hemispheric, Cortical, and Striatal Atrophy as Percentages of Contralateral Side | Atrophy of Contralateral Side, % | NSCs-transplanted | Control | P       |
|---|---|---|---|
| Hemisphere | 21.0±5.3 | 17.0±2.2 | 0.51 |
| Cortex | 4.3±2.6 | 4.4±2.0 | 0.95 |
| Striatum | 25.0±8.0 | 15.5±4.7 | 0.26 |

Mann-Whitney U test was used because the Kolmogorov-Smirnov test showed P<0.05.

Figure 3. Differentiation of NSCs in the perihematomal area. Eight weeks after transplantation, intravenously injected NSCs differentiated into neurons (NeuN and neurofilaments in red) or astrocytes (GFAP in red). Neurofilament-positive cells (d, e, f) and GFAP-positive cells (g, h, i) had a mature morphology similar to that of the host neurons and astrocytes, respectively. Scale bar=20 µm.
genic for transplanted NSCs.\textsuperscript{30} When Ogawa et al\textsuperscript{30} transplanted intraspinally neurosphere-derived neural progenitor cells 24 hours after injury, almost none of grafted cells survived. However, functional improvement was observed and the grafts survived when the cells were transplanted after 9 days. This brief window of opportunity might arise because the microenvironment in the host spinal cord changes rapidly after injury. There might be some differences between the ICH and spinal cord injury models, but common mechanisms such as those involving acute inflammatory changes and hemorrhage exist. Locally injected cells died within 1 day, but intravenously injected NSCs were successfully recruited into the injured brain. Even though many inflammatory cytokines might be neurotoxic or have an astrocyte-inducing effect,\textsuperscript{30} intravenously infused NSCs in our experiment gave rise to functional improvement. These differences might be related to both the administration route and the characteristics of NSCs.

Conclusions

Intravenously transplanted human NSCs enter the rat brain with experimental ICH and survive, migrate, and promote functional recovery. Transplantation of NSCs represents a new therapeutic strategy in ICH and in cerebral ischemia. In vitro expandable NSCs may constitute a good source for neural transplantation. Further studies are needed to elucidate the mechanisms behind the functional benefit derived from NSCs in the experimental ICH model.

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