Survival and Differentiation of Transplanted Neural Stem Cells Derived from Human Induced Pluripotent Stem Cells in A Rat Stroke Model

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Background: Although administration of various stem cells has shown promise in stroke models, neural stem cells (NSCs) derived from human induced pluripotent stem cells (iPSCs) have advantages over other cell types. We studied whether these cells could survive, differentiate, and improve stroke recovery in an ischemic stroke model. *Methods:* Human iPSCs were induced in vitro to an early NSC stage. One week after focal cerebral ischemia, 20 rats received cells or vehicle by intracerebral injection. Graft cell fate, infarct volume, and behavioral deficits were assessed. *Results:* Graft cells were found in 8 of the transplanted rats (80%), with estimated mean graft cell numbers nearly double the amount transplanted 1 month later. Graft cells also expressed markers of NSCs in 5 rats (63%), neurons in all 8 rats (100%), rare astrocytes in 4 rats (50%), and signs of proliferation in 4 rats (50%), but no tumor formation was observed. Stroke volume and behavioral recovery were similar between the groups. *Conclusions:* To our knowledge, this is the first report of transplantation of NSCs derived from human iPSCs in a stroke model. Human iPSC-derived NSCs survived in the postischemic rat brain and appeared to differentiate, primarily into neurons. This cell transplantation approach for stroke appears to be feasible, but further optimization is needed. **Key Words:** Induced pluripotent stem cell—neural stem cell—stroke—transplantation.

Stroke is a major cause of disability, with few effective treatments available to improve recovery.[1] Cell transplantation is a promising potential treatment to improve stroke recovery, and multiple cell types have shown promise in stroke models.[2] Neural stem cells (NSCs) are the precursors of neurons and glia, the primary cells lost with stroke. NSCs may be obtained from embryonic or fetal tissue, but ethical concerns and the potential need for long-term immunosuppression to prevent graft rejection are problems that may limit their utility. NSCs may also be derived from induced pluripotent stem cells (iPSCs), which behave in a similar manner to embryonic stem cells in vitro but are created from adult cells, such as skin fibroblasts. These cells could be obtained from the stroke patient, avoiding both ethical concerns and the need for immunosuppression.[3] We sought to determine if NSCs derived from human iPSCs could survive, differentiate, and improve stroke recovery after transplantation into a rat model of ischemic stroke.

Methods

The human iPS-DF6-9-9T cell line was chosen because it was created from postnatal skin fibroblasts and free of transgenes,[4] and they were differentiated to an early NSC phenotype as previously described,[5] with the following

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modifications. The cells were expanded on a feeder layer of irradiated mouse embryonic fibroblasts with a daily change of embryonic stem cell medium (ESM) that consisted of Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM/F12), 20% serum replacement (Gibco; Invitrogen, Carlsbad, CA), 1% minimum essential medium Eagle: nonessential amino acids (MEM-NEAA; Gibco), 0.1 mM beta-mercaptoethanol (Sigma Chemical, Saint Louis, MO), 1 mM L-glutamine (Gibco), and 4 ng/mL fibroblast growth factor 2 (FGF2; R&D Systems, Minneapolis, MN). Cells were passaged to new plates with 0.1 to 0.2 mg/mL dispase (Gibco) at 37°C for 1 minutes and were mechanically dissociated every 7 days.

The cells were directed in vitro for 21 days to the NSC phenotype of neuroepithelial cells expressing Pax6 and Sox1 but not Oct4. Cell colonies were suspended in ESM without FGF2, and cultured for 4 days in 25 cm^2 tissue culture flasks (Nunclon; Thermo Fisher Scientific, Rochester, NY) with a daily medium change. The cell colonies grew as floating clusters, while any remaining feeder cells adhered to the flask and were removed by transferring the clusters into new flasks. On day 4, the daily medium change was switched to neural induction medium (NIM) of DMEM/F12, 1% MEM-NEAA, 1% N2 supplement (Gibco), and 2 μg/mL heparin (Sigma). On day 7, the clusters were attached to plastic plates with the addition of 10% fetal bovine serum (Gibco) for 12 hours, and some clusters were attached to laminin (Sigma)-coated glass coverslips for immunostaining. Adherent colonies had NIM changed every other day and rosettes formed. On day 14, rosettes were mechanically separated from the surrounding flat cells and again grown as floating clusters in a flask with a change of NIM with 2% B27 supplement (Gibco) every other day. On day 19, the size of the clusters was reduced with accutase and trituration. On day 21, the cluster solution was well mixed and a small portion was immediately removed for counting. The clusters in that portion were dissociated to a single-cell suspension with accutase, and the concentration of live and dead cells was determined using trypan blue stain and a hemocytometer. The clusters were then suspended at a concentration of approximately 50,000 cells per microliter in transplantation medium of NIM, 2% B27, and growth factors (brain-derived neurotrophic factor, glial cell line–derived neurotrophic factor, and insulin-like growth factor 1, each at 1:10,000, and cyclic adenosine monophosphate at 1:1,000). Morphologic analyses and immunostaining with markers for progenitors and more mature neural cells were performed during the course of in vitro differentiation to monitor consistency of differentiation.

All animal procedures were approved by the institutional animal care and use committee. For the procedures, rats were anesthetized with 1% to 2% inhaled isoflurane in oxygen, local bupivicaine 3 mg/kg, and systemic buprenorphine 0.02 mg/kg were given subcutaneously (SC), and body temperature was maintained at 37°C with a rectal probe and heating pad feedback system. Ischemic stroke was induced in adult male Wistar rats (Charles River Laboratories, Charles River, WV) weighing 275 to 325 g by 30 minutes of intraluminal filament middle cerebral artery occlusion (MCAO) as previously described,[6] with modifications. Through a ventral neck incision, the left carotid bifurcation was exposed and the proximal end of the exposed portion of the common carotid artery (CCA) was permanently ligated. The distal CCA was temporarily ligated and an arteriotomy was made between the ligatures for insertion of a synthetic filament with the tip coated with silicone (Doccol Corp, Redlands, CA). The filament was advanced up the internal carotid artery until resistance was felt at 15 to 20 mm distal to the carotid bifurcation indicating MCAO. Anesthesia was maintained during the 30 minutes of MCAO, at which point the filament was removed, the distal CCA permanently ligated, the incision closed, and the rat kept warm during recovery.

Seven days after MCAO, rats were randomly assigned to treatment groups, placed in a stereotactic frame, and a small craniotomy was made at 0 mm anterior and 3 mm left of bregma. Approximately 250,000 cells in 5 μL of vehicle were loaded in a syringe with a 26-gauge needle, which was slowly inserted to a depth of 6 mm from the skull and left in place for 3 minutes before the first injection. Boluses of 1 μL each were injected at 5 depths (6, 5, 4, 3, and 2 mm) relative to the skull at a rate of 1 μL/min, and the needle was left in place 3 minutes after the last injection before removal. The incision was closed and the rat was kept warm during recovery. The control rats had identical injections of vehicle without cells. Cyclosporine 10 mg/kg/day SC was given to all the rats (cell-treated and control) for 7 days starting 2 days before transplantation, followed by 100 μl/ml in drinking water through the survival period.

Behavioral testing was performed, blind to group assignment, twice at each of 4 time points relative to MCAO: before MCAO (baseline) and 1, 3, and 5 weeks after MCAO. The elevated body swing test assessed motor symmetry by gently lifting the rat by the tail and recording the direction of lateral body flexion through 20 trials.[7] The cylinder test assessed symmetry of independent forelimb use as the rat explored the walls of a transparent plastic cylinder for 50 to 100 total touches.[8] The adhesive removal test assessed symmetry of forelimb sensation when small adhesive dots 9.5 mm in diameter were simultaneously placed on both dorsal forepaws, and the difference in removal time was recorded up to a 10-minute trial.[9]

Five weeks after MCAO, rats were anesthetized with isoflurane in oxygen and perfused with saline followed by 4% paraformaldehyde (PFA). The brain was removed and stored in 4% PFA at 4°C for 1 day, followed by phosphate-buffered saline (PBS) with 30% sucrose. After freezing the brain with dry ice, 30-μm coronal sections were made with a sliding microtome through the striatum starting at the anterior corpus callosum. Sections were