Survival and differentiation of neural progenitor cells derived from embryonic stem cells and transplanted into ischemic brain

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Object. Cell replacement therapy including the use of embryonic stem cells (ESCs) may represent a novel treatment for damage from stroke. In this study, the authors transplanted neural progenitor cells (NPCs) derived from ESCs into ischemic brain and analyzed their survival and differentiation.

Methods. Multipotential NPCs were generated from ESCs by using the stromal cell–derived inducing activity method. These cells could differentiate in vitro into neurons, glia, and oligodendrocytes, thus revealing them to be neural stem cells. The NPCs were then transplanted into ischemic brain. At 2 weeks postischemia, the transplanted cells occupied 18.8 ± 2.5% of the hemispheric area; by 4 weeks postischemia, 26.5 ± 4% of the hemisphere. At 4 weeks after transplantation, green fluorescent protein (GFP)-positive transplanted cells showed mature neuronal morphological features. The authors also investigated the expression of differentiation markers and various neurotransmitters. Transplanted cells were immunopositive for neuronal nuclei, β-tubulin-III, and glial fibrillary acidic protein. Of the GFP-positive cells, 33.3 ± 11.5% were positive for glutamate decarboxylase, 13.3 ± 5.8% for glutamate, 2.1 ± 2.5% for tyrosine hydroxylase, 1.8 ± 2% for serotonin, and 0.4 ± 0.2% for choline acetyltransferase.

Conclusions. The authors confirmed the survival and differentiation of ESC-derived NPCs transplanted into the ischemic brain. Surviving transplanted cells expressed several neural markers and neurotransmitters. These findings indicate that these cells can function in the brain.

Key Words • neural progenitor cell • embryonic stem cell • brain ischemia • stroke • mouse

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Materials and Methods

Induction of Neural Differentiation of ESCs

Undifferentiated murine ESCs (G4-2) were maintained on gelatin-coated dishes in Glasgow minimum essential medium (Sigma, St.
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Loui, MO) supplemented with 1% fetal calf serum (JRH Biosciences, Tokyo, Japan), 5% Knockout serum replacement (Invitrogen Corp., Carlsbad, CA), 2 mM glutamine (Sigma), 0.1 mM nonessential amino acids (Invitrogen Corp.), 1 mM pyruvate (Invitrogen Corp.), 0.1 mM 2-mercaptoethanol (Sigma), and 2000 U/ml LIF (Invitrogen Corp.). The G4-2 cells (a kind gift from Dr. Hitoshi Niwa, Osaka University) carry the blastocidin S-resistance selection marker gene driven by the Oct3/4 promoter (active under differentiated status) and the puromycin-resistance selection marker gene driven by the CAG promoter and were maintained in medium containing 20 g/ml blastocidin S (Invitrogen Corp.) and 1.5 g/ml puromycin (Carbiosynth, San Diego, CA) to eliminate differentiated cells. The neurons were induced from murine ESCs by SDIA, as previously described. Briefly, ESCs were cocultured on PA6 stromal cells in Glasgow minimum essential medium supplemented with 5% KnockOut serum replacement, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate, and 0.1 mM 2-mercaptoethanol (differentiation medium). The ESC colonies that formed on PA6 monolayers during 12 days of culture were used for making NPCs and were isolated by incubation with papain for 5 minutes at room temperature (Papain Dissociation System; Worthington Biochemical Corp., Lakewood, NJ). Isolated colonies were cultured in neurobasal medium (Invitrogen Corp.) with B27 supplement (Invitrogen Corp.), 20 ng/ml fibroblast growth factor 2 (Upstate Biotechnology, Waltham, MA), 20 ng/ml epidermal growth factor (R&D Systems, Minneapolis, MN), and 10 ng/ml LIF for 1 week. The NPCs derived from the ESCs were collected and used for transplantation.

Immunofluorescence Study

Single- and double-immunofluorescence studies were performed after permeabilization and blocking for nonspecific binding with 0.3% Triton X-100 (Sigma) and 10% normal donkey serum (Jackson Immunoresearch Laboratories, Inc., West Grove, PA), respectively. Primary rabbit anti-TUJ antibody (1:60; Chemicon, Temecula, CA), mouse anti-TUJ antibody (1:300; Convance Research Products, Richmond, CA), rabbit anti-GFAP antibody (1:1000; Chemicon), and anti-CNPase (1:200; Chemicon) were used at 4˚C overnight; donkey fluorescein isothiocyanate-labeled secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) or donkey fluorescein isothiocyanate-labeled secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) were used at room temperature for 120 minutes. Green fluorescent protein, contained in the ESCs, was visualized by its own fluorescence. The percentage of immunopositive cells after papain treatment was evaluated using a laser confocal microscope (Fluoview FV300; Olympus Optical Co., Tokyo, Japan).

Induction of Focal Ischemia

The C57BL/6 mice (20-25 g) were anesthetized with 2% isoflurane and anesthesia was maintained with 1.5% isoflurane in 70% N2O/30% O2. Regional cerebral blood flow was measured as described using a laser Doppler flowmeter (Ohiogawa, Tokyo, Japan) equipped with a flexible skull probe. The tip of the probe was fixed using an adhesive agent and its accelerator (Aron Alpha; Toa, Tokyo, Japan) on the intact skull over the ischemic cortex (2 mm posterior and 6 mm lateral from bregma). Steady-state baseline values were recorded before MCA occlusion, and rCBF during and after occlusion was expressed as a percentage of the baseline values. Rectal temperature was maintained between 36.5 and 37.5 C with a homeothermic blanket. Focal cerebral ischemia was induced by the intraluminal filament technique. The left MCA was occluded using a No. 8-0 nylon monofilament (Ethicon, Somerville, NJ) coated with a mixture of silicone resin (Xantopren; Bayer Dental, Osaka, Japan) and a hardener (Elastomer Activator; Bayer Dental). This coated filament was introduced into the internal carotid artery through the common carotid artery, up to the origin of the anterior cerebral artery via the internal carotid artery, to occlude the MCA and posterior communicating artery. The animals that demonstrated a more than 80% reduction in rCBF from baseline were included in this study. Thirty minutes after MCA occlusion, while the mice were kept at 37 C by using a blanket, the filament was withdrawn from the common carotid artery to allow reperfusion.

Results

Neural Progenitor Cells Generated From ESCs

The SDIA method was originally created for making dopaminergic neurons. With this method, ESCs are routinely maintained on gelatin-coated dishes (Fig. 1a). When cultured on a monolayer of PA6 cells, however, they differentiate into NPCs within 10 days and into dopaminergic neurons within 14 days. Thus, we cultured ESCs on PA6 cells for 10 days and made neurospheres consisting of NPCs. These cells were positive for neural cell adhesion molecule, which is a marker of neuroprogenitors (Fig. 1b). The ESC-derived NPCs formed round neurospheres cultured in the medium (Fig. 1c). These cells could differentiate in vitro into neurons (TuJ1-positive; Fig. 1d and e), glia (Fig. 1d and e), and oligodendrocytes (Fig. 1f), thus indicating them to be neural stem cells.

Physiological Data and Infarct Volume

All animals that underwent surgery demonstrated a more than 80% reduction in rCBF from baseline and were included in this study. The mean rCBFs during ischemia and after reperfusion were 8.5 ± 3.6 and 102.2 ± 18.4%, respectively (five-eight mice per group; preoperative CBF was set as

Infarct Volume

The infarct volume was calculated by summing the volumes of each section directly by using the NIH image analyzing system (Dr. Wayne Rashband, National Institutes of Health, Bethesda, MD). The data were statistically analyzed using the unpaired t-test.

Cell Transplantation

While in a state of deep pentobarbital anesthesia, the mice were placed in a stereotactic frame (Narisige, Tokyo, Japan) 2 days after ischemia/reperfusion. Each animal received a 2-μl injection (1 μl/mm) of neurospheres derived from ESCs into the lesioned striatum (from the bregma: anterior 3.5, lateral ± 3, ventral 0) via a Hamilton microsyringe fitted with a 26-gauge blunt needle.

Immunohistochemical Analysis

Fourteen (four animals) or 28 days (four animals) after transplantation, animals were killed with pentobarbital and perfused transcardially with PBS, followed by 4% paraformaldehyde. The mouse brains were postfixed overnight and equilibrated in graded sucrose. Coronal 40-μm sections were cut serially on a freezing microtome and postfixed in 4% paraformaldehyde for 10 minutes. After several washes in 0.1 M PBS, the sections were incubated for 1 hour at room temperature with 10% normal goat serum (Vector Laboratories, Burlingame, CA) containing 0.3% Triton X-100 (Sigma). Immunohistochemical staining was performed at 4 C for 16 hours using antibodies against NeuN (1:200; Chemicon), TuJ1 (1:300; Convance Research Products, GFAP (1:150; Chemicon), GAD (1:100), glutamate (1:200; Chemicon), ChAT (1:100; Chemicon), TH (1:60; Chemicon), serotonin (1:200; Chemicon), with 2% normal goat serum, 0.3% Triton X-100, and 0.1% NaN3 in PBS used as the diluent. After three rinses in PBS, the sections were incubated for 1 hour at room temperature with Cy3-labeled (Jackson Immunoresearch Laboratories, Inc.) or fluorescein isothiocyanate-labeled secondary antibodies (Jackson Immunoresearch Laboratories, Inc.) and were evaluated using a confocal microscope. Grafted ESCs were detected by their green fluorescence. The area of immunopositive cells was quantified in every fourth section throughout the graft and its surroundings.
Infarct volume at 2 and 4 weeks after transplantation was 9.82 and 8.25 mm, respectively (four mice per group). Note that the difference between the groups was not significant.

Transplantation of ESC-Derived NPCs Into Ischemic Brain

Thirty minutes of MCA occlusion resulted in brain infarction, which was restricted to the lateral striatum. Cells transplanted into the ischemic brain were located widely around the ischemic area (Fig. 2a–f). At 2 weeks after ischemia, the transplanted cells occupied 18.8 ± 2.5% of the hemispheric area (Fig. 2a–c and Table 1). By 4 weeks posts ischemia, this occupation increased to 26.5 ± 4% (Fig. 2d–f). On the contrary, in sham-operated animals, ESC-derived cells moved laterally along the corpus callosum. As
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shown in Fig. 2, surviving cells occupied a small area (Fig. 2g–i). Infarct volumes were not significantly different between the specimens prepared 2 weeks after ischemia and those 4 weeks after ischemia (data not shown).

Differentiation of Transplanted ESC-Derived NPCs

At 4 weeks after transplantation, GFP-positive cells demonstrated mature neuronal morphological features (Fig. 3a). In addition, we investigated the expression of both the differentiation markers and the various neurotransmitters (Fig. 3 and Table 2). Transplanted cells were immunopositive for NeuN (Fig. 3b), TuJ1, and GFAP (Fig. 3c). Of the GFP-positive cells, 60 ± 10% were NeuN-positive, 40 ± 10% TuJ1-positive, and 22 ± 7.2% GFAP-positive. Only 0.4 ± 0.5% of the GFP-positive cells were GalC-positive. Next we examined neurotransmitter expression of the transplanted cells. Of the GFP-positive cells, 33.3 ± 11.5% were GAD-positive (Fig. 3d), 13.3 ± 5.8% glutamate-positive (Fig. 3e), 2.1 ± 2.5% TH-positive (Fig. 3g), 1.8 ± 2% serotonin-positive (Fig. 3h), and 0.4 ± 0.2% ChAT-positive (Fig. 3e) cells.

Discussion

In this study, we generated multipotential NPCs from ESCs and transplanted them into ischemic mouse brain. These cells could survive and expand widely in the ischemic area. Moreover, the transplanted NPCs differentiated into various types of neural cells.

<table>
<thead>
<tr>
<th>Marker</th>
<th>% GFP</th>
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<tbody>
<tr>
<td>NeuN</td>
<td>60.0 ± 10.0</td>
</tr>
<tr>
<td>TuJ1</td>
<td>40.0 ± 10.0</td>
</tr>
<tr>
<td>GFAP</td>
<td>22.0 ± 7.2</td>
</tr>
<tr>
<td>GalC</td>
<td>0.38 ± 5.3</td>
</tr>
<tr>
<td>TH</td>
<td>0.6 ± 0.36</td>
</tr>
<tr>
<td>GAD</td>
<td>33.3 ± 11.5</td>
</tr>
<tr>
<td>Glutamate</td>
<td>13.3 ± 5.8</td>
</tr>
<tr>
<td>Serotonin</td>
<td>0.5 ± 0.44</td>
</tr>
<tr>
<td>ChAT</td>
<td>0.4 ± 0.2</td>
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* Data are expressed as the means ± standard deviation.
Embryonic stem cells have many characteristics required for an optimal source for cell replacement therapy. The ESCs are self-renewing, multipotent cells derived from the inner cell mass of the preimplantation blastocyst. Kawasaki and colleagues previously reported a strong neuralization-inducing activity present on the cell surface of stromal cells and named it “SDIA.” In the absence of exogenous BMP4, mouse ESCs were shown to differentiate efficiently into NPCs and neurons when cultured on SDIA-possessing mouse stromal cells (PA6 cells) for 1 week. Recently, the SDIA method has also become applicable for primate ESCs. After having been cultured on PA6 cells for 2 weeks, the majority of primate ESC colonies contained a large number of NPCs and postmitotic neurons. Neural progenitor cells have multipotent, self-renewing capacities and can be cultured as neurospheres.

In this study, we analyzed several neurotransmitters’ expressions. Note that GAD is a γ-aminobutyric acid synthetic enzyme. The γ-aminobutyric acidergic neurons in the cerebellum are rich in ventral mesencephalon or Purkinje cells. Choline acetyltransferase is a synthetic enzyme of acetylcholine. Cholinergic neurons are rich in the basal forebrain and associated with Alzheimer disease. Glutamate is used by descending pathways originating from neocortical pyramidal cells. The dorsal raphe nucleus is known to be rich in serotonin, which is implicated in emotion, fear, and cognition. Dopaminergic neurons of the substantia nigra are lost because of Parkinson disease. Tyrosine hydroxylase expression is linked to the secretion of levadopa, which is a dopamine precursor. To determine cell types, we used several markers. Beta-Tubulin-III is expressed in postmitotic neurons at an early stage of development. The NeuN is an nuclear protein that is a marker of a mature neuron. Both GalC and CNPase are thought to be mature oligodendrocyte markers. As an astrocyte marker, GFAP is detectable during fetal glial development.

The use of neural transplantation for the treatment of neurological diseases first became a potential therapeutic modality in 1979 when Bjorklund and colleagues demonstrated that implanting dopaminergic-containing neurons...
into the rat striatum improved functional deficits induced by damage to the nigrostriatal pathway. Since then, advances in neural transplantation have moved from the animal model to the human model, with varying degrees of success.\textsuperscript{1,11,19,20,23,29} In the animal models, authors examined a wide variety of disease states—from degenerative diseases to trauma and stroke—and the tissues used for transplantation—from fetal tissue to tumor lines to stem cells.\textsuperscript{19,20,22,23,26,29} In some models, implants provide a source of neurotrophic factors.\textsuperscript{4,12} Successes in animal models have led to transplant trials in the human population. Patient trials have been focused on transplantation for Parkinson disease, Huntington disease, spinal cord injury, and stroke.\textsuperscript{4,11} As research in animal models progresses, transplant trials may be initiated for the treatment of multiple sclerosis, traumatic brain injury, cerebral palsy, amytrophic lateral sclerosis, Alzheimer disease, and other disorders.\textsuperscript{11,13,20}

In patients disabled by stroke, the concept of restoring function by transplanting human neuronal cells into the brain is a novel one. Data obtained from a rat model of transient focal cerebral ischemia demonstrated that transplantation of fetal tissue restored both behavioral and motor functions.\textsuperscript{17,19,20,29} As for studies in humans, Kondziolka and colleagues\textsuperscript{14,18} reported the results of a clinical trial using human neuronal cells. In examining 12 patients in this trial, their initial objective was to demonstrate the safety and feasibility of the neuronal cell implantation procedure. Among the treatment groups, mean National Institutes of Health Stroke Scale Total scores decreased and mean European Stroke Scale Total scores increased—both changes indicating improvement.\textsuperscript{14,18} The transplanted cells were proposed to have improved neurological function through a number of different mechanisms, including provision of neurotrophic support, production of neurotransmitters, reestablishment of local interneuronal connections, cell differentiation and integration, and improvement of regional O$_2$ tension.

In the present study, we used ESC-derived NPCs for transplantation. The advantage in ESCs is that they can be expanded easily compared with neural stem cells. We also confirmed the differentiation of ESC-derived NPCs. During ischemia, various types of neurons as well as glial cells and oligodendrocytes are lost. The ESCs could supply these cells. Interestingly, in the sham-operated control brains, the transplanted cells occupied only a small area. On the contrary, in the ischemic brain, the transplanted cells spread throughout the ischemic lesion. This result indicates that the fate of the graft is dependent on the host environment. After ischemia, several cytokines and growth factors are known to be released. Today, the family of growth and trophic factors has been proposed to affect the survival and development of neuroprogenitor cells. Among them, LIF and ciliary neurotrophic factor in addition to more traditional growth factors, such as platelet-derived growth factor, are considered to be potent promoters of neuroprogenitor cell proliferation and their eventual differentiation.\textsuperscript{1,2} Moreover, brain-derived neurotrophic factor, another member of the neurotrophin family (which includes nerve growth factor, neurotrophin-3, and neurotrophin-4/5), was shown to have great potency in modulating the growth and survival of dopaminergic cells and their precursors.\textsuperscript{1,2} Gliad-derived neurotrophic factor has similar or even enhanced trophic effects on dopaminergic neurons and their precursors.\textsuperscript{1,2}

In using a 30-minute ischemia model, we did not examine behavioral improvement after transplantation, because such a model demonstrates only a slight behavioral deficit, thus making it difficult to assess behavior. Therefore we will use a longer period of ischemia in the next study and will examine network formation.

Conclusions

In summary, we confirmed the survival and differentiation of ESC-derived NPCs transplanted into the ischemic brain. We used the SDIA method on murine ESCs. Note that this method is also effective on primate and even human ESCs. Our findings indicated that ESC-derived NPCs can function in the brain.

References


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