Transplantation of Human Embryonic Stem Cell-Derived Cells to a Rat Model of Parkinson’s Disease: Effect of In Vitro Differentiation on Graft Survival and Teratoma Formation

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ABSTRACT

Human embryonic stem cells (hESCs) have been proposed as a source of dopamine (DA) neurons for transplantation in Parkinson’s disease (PD). We have investigated the effect of in vitro predifferentiation on in vivo survival and differentiation of hESCs implanted into the 6-OHDA (6-hydroxydopamine)-lesion rat model of PD. The hESCs were cocultured with PA6 cells for 16, 20, or 23 days, leading to the in vitro differentiation into DA neurons. Grafted hESC-derived cells survived well and expressed neuronal markers. However, very few exhibited a DA neuron phenotype. Reversal of lesion-induced motor deficits was not observed. Rats grafted with hESCs predifferentiated in vitro for 16 days developed severe teratomas, whereas most rats grafted with hESCs predifferentiated for 20 and 23 days remained healthy until the end of the experiment. This indicates that prolonged in vitro differentiation of hESCs is essential for preventing formation of teratomas.

INTRODUCTION

Human embryonic stem cells (hESCs) can potentially be used for cell replacement therapy in Parkinson’s disease (PD) [1, 2]. Differentiation into dopamine (DA) neurons is necessary prior to their use for transplantation. The optimal in vitro differentiation protocol should produce a sufficient number of committed DA progenitors and eliminate pluripotent cells that can give rise to teratomas.

Several culture conditions direct ESCs toward differentiation into DA neurons [3–6]. Coculturing with the PA6 stromal cell line rapidly generates high numbers of DA neurons from mouse and monkey ESCs by an unknown mechanism named stromal-derived inducing activity (SDIA) [3, 7]. These DA neurons can survive transplantation, integrate into the host striatum, and reduce functional deficits in animal models of PD [3, 7–11]. The same protocol induces DA cell fate in cultures from hESCs, but the efficacy varies between different hESC cell lines [12, 13]. Furthermore, the survival of hESC-derived DA neurons after transplantation is low and no functional benefit from these grafts has been reported [12, 13].

Results concerning teratoma formation from grafted SDIA-derived ESCs are controversial. Thinyane et al. reported the development of teratomas 5 weeks after transplantation of mouse ESCs first differentiated on PA6 cells in vitro [9]. In
contrast, no teratoma formation was detected in studies performed on grafted hESCs that had been predifferentiated on PA6 cells [12, 13].

In the current study, we characterized the differentiation of hESCs (line SA002.5) into DA neurons when cocultured with PA6 cells in vitro and examined their survival after grafting into the striatum of a rat model of PD. The hESCs were transplanted after 16, 20, or 23 days of differentiation in coculture. Changes in lesion-induced behavioral deficits were monitored. Survival, neuronal differentiation, and teratoma formation were assessed 2 and 13 weeks after transplantation.

MATERIALS AND METHODS

SDIA-Mediated Differentiation of hESCs into DA Neurons

Undifferentiated hESCs of line SA002.5 (Cellartis, Gothenburg, Sweden, http://www.cellartis.com) were maintained as previously described [14]. PA6 cells were cultured as described elsewhere [7]. For differentiation experiments, PA6 cells were plated on type I collagen-coated chamber slides or 0.1% gelatin-coated Petri dishes (diameter, 6 cm) at confluence 1 day before introducing hESCs into the coculture. The hESCs were dissociated after 15–30 minutes of incubation in type IV collagenase (200 U/ml; Sigma, St. Louis, http://www.sigmaaldrich.com) and plated on PA6 cells at the density of 2 × 10⁴ cells per cm² in ESC differentiation medium (ESCDM) that consisted of Glasgow’s modified Eagle’s medium (Gibco, Grand Island, NY, http://www.invitrogen.com) supplemented with 8% Knockout Serum Replacement (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 1 mM pyruvate (Sigma), 0.1 mM nonessential amino acids (Gibco), and 0.1 mM 2-mercaptoethanol (Gibco). From day 6 onward, half of the medium was replaced every 3rd day. Two weeks after plating, colonies were separated from the feeder using a papain dissociation kit (Worthington Papain Dissociation System, New Jersey, USA, http://www.worthingtonbiochem.com) with 100,000 viable hESC-derived cells (2 μl of cell suspension) cultured for 16 (group 1, n = 22), 20 (group 2, n = 8), or 23 days (group 3, n = 8) on PA6 cells. Cells that were not implanted were seeded on PORN/laminin-coated eight-well glass chamber slides (Falcon, Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bdb.com) containing ESCDM.

Electrophysiological Recordings

Three to 4 weeks after plating hESCs on PA6 cells, the cultures were placed on an upright Nikon E600FN microscope (Nikon Corporation, Tokyo, http://www.nikon.com) and continuously superfused with a solution (bubbled with 95% O₂−5% CO₂ [pH ~7.4]) containing 124 mM NaCl, 3 mM KCl, 1.6 mM CaCl₂, 2.4 mM MgCl₂, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, and 10 mM d-glucose. Whole-cell patch-clamp recordings were performed on 61 hESCs (from six different cultures) identified using infrared-differential interference contrast video microscopy. Patch pipette resistances were 2–8 MΩ, and series resistance varied between 5 and 25 MΩ. Recordings were made in current-clamp or voltage-clamp mode using an EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany, http://www.heka.com). Data were sampled at 20 kHz and filtered at 3 kHz. A series of 10 hyperpolarizing and depolarizing current pulses (40–140 pA, 500-ms duration) was applied when the membrane potential was held at −60 and −80 mV.

Electromicroscopy

Cultures differentiated for 3 weeks were fixed in 0.1 M PBS with 2.5% glutaraldehyde, 2% paraformaldehyde (PFA), 0.05% sodium azide, and 0.05 M sodium cacodylate. Cells were post-fixed in OsO₄ and dehydrated in ethanol before embedding in epoxy resin. Ultrathin sections (60–70 nm) were cut on a Reichert Ultramicrotome (Reichert Microscope Services, Depew, NY, http://www.reichertms.com), contrasted with lead citrate and uranyl acetate, and examined in a LEO 912AB transmission electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany, http://www.leo-em.com) equipped with a Proscan and a Megaview III camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany, http://www.soft-imaging.net).

6-Hydroxydopamine Lesions and Transplantation of hESC-Derived DA Neurons

Female Sprague-Dawley rats (B&K Universal Ltd., Sollentuna, Sweden, http://www.bluw.com) weighing 180–200 g received two stereotactic injections of 6-hydroxydopamine (6-OHDA) into the right nigra-striatal pathway [15].

Differentiated hESCs that had been grown on PA6 cells for 16, 20, or 23 days were separated from the PA6 cells using the Worthington Papain Dissociation System. (See supplemental online Fig. 1 for the detailed experimental scheme.) They were then dissociated into a single-cell suspension using a pipette after incubation in Accutase cell detachment solution (Innovative Cell Technologies, Inc., San Diego, http://www.innovativetechnology.com). The cell suspension contained 50,000 viable cells per μl (assessed by trypan blue) in Hanks’ balanced saline solution with 0.05% DNase. The right striatum of each rat was stereotactically implanted using a 10-μl Hamilton microsyringe (Hamilton Company, Reno, NV, http://www.hamiltoncomp.com) with 100,000 viable hESC-derived cells (2 μl of cell suspension) cultured for 16 (group 1, n = 22), 20 (group 2, n = 8), or 23 days (group 3, n = 8) on PA6 cells. Injection coordinates (in mm) were anterior = +1.0; lateral = +3.0; ventral = −4.5 and −5.0 [16]. All rats were immuno-suppressed with intraperitoneal injections of 15 mg/kg of Cy closporine A given 1 day prior to transplantation, daily for 2 weeks after transplantation, and at 10 mg/kg per day thereafter. Cells that were not implanted were seeded on PORN/laminin-coated eight-well chamber slides (14,000 cells/well) and fixed 24 hours later. To assess ongoing cell proliferation, each rat was injected with 5-bromo-2′-deoxyuridine (BrDU) (50 mg/kg, i.p.) that incorporates into the DNA in the S phase of the cell cycle. To label most of the dividing cells in the grafts, BrDU was injected three times at 8-hour intervals 24 hours prior to sacrifice.

Amphetamine-Induced Rotations

Three to 4 weeks after the 6-OHDA lesion, the rats received an injection of d-amphetamine (2.5 mg/kg, i.p.). Motor asymmetry was monitored in automated “rotometer bowls” for 90 minutes [17]. Rats displaying rotational scores of more than five net ipsilateral turns (consistent with a complete (>98%) reduction of striatal DA) were selected for transplantation. The rotation test was repeated at 2, 4, 8, and 13 weeks after grafting.
Immunohistochemistry and Cell Quantification

Immunostained cell cultures prepared for the study of SDIA-mediated differentiation of hESCs into DA neurons were visualized by a Zeiss fluorescent microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com) attached to a Nikon digital camera. The total number of colonies and the number of β-III-tubulin- and tyrosine hydroxylase (TH)-positive colonies were counted. A colony was defined as “immunoreactive” if more than approximately 10% of the cells were stained by the marker in question. In some randomly chosen colonies, the proportions of TH/β-III-tubulin-immunoreactive cells were evaluated (60–200 cells/colony, approximately 1,800 cells/group).

Rats were perfused with 4% PFA. The brains were dissected and post-fixed with the same fixative for 24 hours, and 40-µm thick coronal sections were cut using a microtome. Immunostaining was performed on free-floating sections. (See supplemental online Table 1 for information of antibodies used.) Immunostained brain sections and cell cultures were analyzed under a fluorescent microscope (Olympus BX60; Olympus, Tokyo, http://www.olympus-global.com). Double-stained cells were visualized by confocal microscopy (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany, http://www.leica.com). Cell numbers were assessed using an Olympus CAST-Grid system (Olympus). Surface areas in sections covered by grafted human nuclei (HNuc)-positive cells were delineated. Using a systematic random sampling technique, the numbers of HNuc- and BrDU- or neuronal nuclei (NeuN)- or TH-positive cells were assessed in 1% of the areas. The total numbers of cells within the grafts were estimated by the equation \[ N = n \times x \times yA(f) \times 10, \] where \( N \) is total number of cells, \( n \) is number of cells in one series, \( x \) is \( x \)-step length, \( y \) is \( y \)-step length, and \( A(f) \) is frame area.

Detection of DA Release by High-Performance Liquid Chromatography

For assessment of DA production, hESCs cocultured with PA6 cells for 16, 20, and 23 days, in similar conditions as the cultures prepared for transplantation, were sonicated in perchloric acid. The supernatant was collected, filtered, and injected into a high-performance liquid chromatography (HPLC) (ESA Coulombex III; ESA Biosciences, Inc., Chelmsford, MA, http://www.esainc.com) for the electrochemical detection of DA. Mobile phase (sodium acetate 5 g/L, octanesulfonic acid 100 mg/L, EDTA 30 mg/L, methanol 12% [pH 4.2]) was delivered at a flow rate of 500 l/minute to a reverse-phase C18 column (4.6 mm diameter, 150 mm length, Chrompack). Peaks were quantified by Azur Chromatographic software (DATALYS, Theix, France, http://www.datalys.net).

Statistical Analysis

Effects of time in culture on hESC differentiation into DA neurons was examined using one-way analysis of variance and Scheffé post hoc test. In the transplantation studies (i.e., survival, proliferation, and neuronal differentiation), statistical analyses were performed using the nonparametric Kruskal-Wallis test. Post hoc pairwise analysis between two groups at a same time point or between two time points within the same group were performed using the nonparametric Mann-Whitney \( U \) test.
RESULTS

In Vitro Differentiation of hESCs
The hESCs were cultured on PA6 cells for 2 weeks and then transferred to PORN/laminin-coated chamber slides for another 0.5, 2, and 4 weeks in vitro. At 2.5 weeks in culture, 42% ± 20% (mean ± SD) of the colonies expressed β-III-tubulin, and 38% ± 22% were positive for TH (Fig. 1A). The percentages of β-III-tubulin- and TH-positive colonies, as well as the percentages of β-III-tubulin- and TH-positive cells within each colony (Fig. 1B), did not change significantly over time. After 6 weeks in culture, only 1.0% ± 2.0% of TH-positive cells were immunoreactive for peripherin, a marker for neurons in the peripheral nervous system, indicating that the vast majority of the generated TH-positive neurons had a central nervous system phenotype (supplementary online Fig. 2).

In whole-cell patch-clamp-recordings carried out after 3–4 weeks of culture in vitro, 26% of the investigated cells showed a mature response pattern with overshooting and generally multiple action potentials (APs) (Fig. 1I). In contrast, 48% of these cells responded with one or two APs with a low amplitude, representative of immature neurons (Fig. 1J) [18]. The electron micrographs showed synaptic contacts between the cultured hESCs (Fig. 1K). Consistent with these findings, we observed frequent spontaneous synaptic responses in one of the cells exhibiting immature APs (Fig. 1L).

Transplantation of SDIA-Mediated Differentiated hESCs

Functionality of DA Neurons Derived by the SDIA Method.
Release of DA was detected by HPLC in supernatant from PA6/hESC cocultures grown in vitro for 20 and 23 days, but not from those grown for only 16 days (Fig. 1M). Hemiparkinsonian rats with 6-OHDA lesions, transplanted with differentiated hESCs, were subjected to the amphetamine-induced rotation test. No significant changes in rotation scores were seen in any of the three groups of transplanted rats at 2, 4, 8, and 13 weeks after transplantation (Fig. 2), indicating that the grafts were not functional.

Survival, Proliferation, and Tumor Formation.
Supernumerary hESC-derived cells harvested before transplantation surgery were replated and grown in vitro. Some of them expressed both β-III-tubulin and TH, indicating that they had survived the dissociation procedure. The proportion of TH-immunopositive cells increased over the time between 16 and 23 days of coculturing with PA6 cells (Table 1).

In the grafts, hESC-derived cells were detected at 2 and 13 weeks after transplantation by immunostaining for HNuc (Fig. 3A). At 2 weeks after transplantation, four rats from each group were sacrificed. Stereological analysis of HNuc-positive cells revealed that cells differentiated for 23 days did not survive the grafting procedure as well as the cells cultured for 16 days (p < .05). Some grafts contained cell numbers (in the range of 250–800 × 10³) by far exceeding the number of implanted cells, suggesting proliferation after grafting. All rats of group 1 and two out of four rats of group 2 had been lost because of teratoma formation at week 13 after transplantation.

<table>
<thead>
<tr>
<th>Culturing time on PA6 feeder cells</th>
<th>16 days</th>
<th>20 days</th>
<th>23 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of β-III-tubulin-positive cells/total cells</td>
<td>8.8</td>
<td>31.9</td>
<td>20.0</td>
</tr>
<tr>
<td>Percentage of TH-positive cells/β-III-tubulin-positive cells</td>
<td>1.9</td>
<td>13.7</td>
<td>37.1</td>
</tr>
<tr>
<td>Percentage of TH-positive cells/total cells</td>
<td>0.2</td>
<td>4.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Abbreviation: TH, tyrosine hydroxylase.

To assess ongoing cell proliferation, each rat was injected with BrdU prior to sacrifice. At 2 weeks after transplantation, all grafts contained considerable numbers of BrdU-positive cells (group 1, 30%; group 2, 6%; group 3, 12% of HNuc-positive cells, median values; Fig. 3D). By 13 weeks, the proportion of HNuc-positive cells that colabeled for BrdU had decreased, indicating that the proliferative capacity declined over time (2% in groups 2 and 3, median values; Fig. 3D).

The tendency to form teratomas differed between the three groups (Fig. 3H). Between 6 and 11 weeks after transplantation, all rats in group 1 developed teratomas with classic morphological features (Fig. 3I, 3J) and had to be sacrificed. In group 2, two rats were lost due to teratoma formation after 12–13 weeks. No teratoma was found in rats from group 3. In line with this, Oct-3/4-positive cells were found in grafts from groups 1 and 2 at 2 weeks after transplantation, but not in grafts from group 3 (supplemental online Fig. 3A–3C). In contrast, grafts in some rats in all three groups still expressed the endodermal marker α-fetoprotein (Fig. 3K, 3L).
Neuronal Differentiation in the Graft. Two weeks after implantation, the proportions of HNuc-positive cells that expressed the neuronal marker NeuN did not differ between the three grafted groups (Fig. 4A). Disregarding rats that developed teratomas, the proportion of grafted cells that expressed NeuN showed similar numbers at all time points after surgery (Fig. 4A). Only 10 to 50 TH-positive cells were found in each graft (Fig. 4E–4J), and these numbers did not differ systematically between the three groups. Furthermore, we examined sections from grafted brains to see whether the hESC-derived cells stained for other neural markers. None of the examined transplanted cells were immunolabeled for NG2 (oligodendrocytes; Fig. 4K), GAD (GABAergic neurons), 5-HT (serotonergic neurons), and ChAT (cholinergic neurons; Fig. 4L), whereas some host cells were immunoreactive for these markers.
DISCUSSION

We show that hESC-derived cells can differentiate into neurons that can generate AP, form synapses at the ultrastructural level, and are capable of synthesizing and releasing DA in vitro. After transplantation, the hESC-derived DA neurons either did not survive in sufficient numbers or they lost their capacity to produce and release DA (Fig. 2). The percentage of DA neurons is comparable with results obtained with SDIA for primate ESCs [3] and for the hESC line HSF-6 [13]. After grafting, only low numbers of TH-positive cells were observed, in agreement with the lack of behavioral recovery in the transplanted animals. This is consistent with previous reports in which very few [12, 14].


REFERENCES


CONCLUSION

The SDIA method efficiently induces neuronal and DA cell fate in hESCs. The risk of teratoma formation after transplantation is significantly decreased with differentiation time in vitro. The fact that no tumors, but a substantial number of neurons, were detected in animals transplanted with hESCs differentiated for 23 days is appealing. This prompts additional protocol optimizations in future studies focusing on in vitro predifferentiation for either direct transplantation of hESC-derived neural cells or for the generation of neuronal progenitors. Furthermore, reasons for the low frequency of TH-positive cells in the grafts need to be understood and counteracted. Importantly, our data support the statement that safety issues in hESC application for transplantation therapy of PD (such as a risk for teratoma formation) should be fully apprehended before approaching the stage of actual clinical trials.

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DISCLOSURES

The authors indicate no potential conflicts of interest.


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