Original Article

Human Retinal Pigment Epithelial Cell Implants Ameliorate Motor Deficits in Two Rat Models of Parkinson Disease

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Abstract

Intrastratal transplantation of gelatin microcarrier-attached human retinal pigment epithelial cells (hRPE-GM) may represent an alternative source for cell therapy in Parkinson disease (PD). The use of human retinal pigment epithelial (hRPE) cells in PD relies on the capacity of these cells to produce l-dopa as an intermediate product in the eumelanin synthesis pathway. We investigated the behavioral effects of hRPE-GM implants on forelimb use asymmetries and hindlimb motor deficits in unilateral and bilateral 6-hydroxydopamine (6-OHDA) rat models of PD. We report that intrastratal unilateral implantation of hRPE-GM in rats with 6-OHDA nigrostriatal lesions produce an amelioration of the contralateral forelimb disuse and the contralateral hindlimb deficits. These results further support the possibility that implantation of cultured hRPE cells may be a promising therapeutic option for patients with PD.

Key Words: Basal ganglia, Parkinsonism, Retinal pigment epithelium, Striatum, Transplantation.

INTRODUCTION

Parkinson disease (PD) is the most common neurodegenerative movement disorder (1) and is characterized by degeneration of the dopaminergic neurons in the substantia nigra pars compacta, accompanied by decreases in striatal dopamine (DA) (2) and the appearance of intracytoplasmic Lewy body inclusions. Once striatal DA loss reaches the 80% critical value (3), a progressive motor impairment develops that is characterized by resting tremor, rigidity, bradykinesia, hypokinesia, and postural instability (4).

A small clinical trial in patients with PD has recently suggested that human retinal pigment epithelial (hRPE) cells may represent an alternative source for cell therapy in PD (5). The retinal pigment epithelium is a pigmented, melanin-containing cellular monolayer that lies between the neural retina and the choroid (6). The use of hRPE cells in PD relies on the capacity of these cells to produce the pigment eumelanin (7). L-Dopa is produced as an intermediate in the eumelanin synthesis pathway, from the metabolism of tyrosine through the action of tyrosinase, a tyrosine hydroxylase-like enzyme (8–10). L-Dopa can be excreted by the cell or further metabolized into dopaquinone, the next step in melanin synthesis.

hRPE cells can be harvested from fetal or neonatal donor eyes, screened for a variety of viruses, endotoxins, and mycoplasms, and multiplied in standard cell culture conditions to produce sufficient cells for multiple patients. hRPE cells are anchorage-dependent and do not survive alone (11), but, like many epithelial cells, hRPE cells attached to gelatin microcarriers (hRPE-GM) display increased survival and no need for immunosuppression (12). Striatal hRPE-GM implants have been shown to ameliorate parkinsonian motor deficits in humans (13, 14), nonhuman primates (15, 16), and rats (11) without adverse effects.

In a study by Subramanian et al (11), the behavioral effects of hRPE-GM implants in unilaterally 6-hydroxydopamine (6-OHDA)-lesioned rats were assessed over an 18-week period using the apomorphine-induced rotation paradigm. This model, although widely used for pharmacologic testing, has the disadvantage that repeated intermittent exposure to the drug may lead to unknown effects of the drug on hRPE cells that possess a DA D2 receptor. Recently, questions have been raised as to the reliability of the model. To avoid some uncertainties of the drug-induced rotation model, we elected to use 2 tests of spontaneous sensorimotor function that are independent of learning, practice, handling or motivation, and evaluate normal spontaneous rodent behaviors such as rearing and walking (17, 18). We used these tests in 2 models of 6-OHDA-induced lesions: a severe unilateral lesion model and a model of moderate bilateral lesion, both with unilateral hRPE-GM implants.

In this study we investigated the behavioral effects of hRPE-GM implants on forelimb use asymmetry and hindlimb motor deficits in a unilateral and a bilateral rat model of PD. We report here that intrastratal unilateral implantation...
of hRPE-GM in rats with 6-OHDA nigrostriatal lesions produces an amelioration of the contralateral forelimb disuse and the contralateral hindlimb deficits. Preliminary results have been reported in abstract form (19).

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (Animal Care Centre Breeding Unit, South Campus, University of British Columbia) weighing 275–300 g at the beginning of the experiments were used. Animals were housed in pairs in Plexiglas cages with free access to rat chow and water under a 12:12-hour light-dark cycle (lights off from 1200 to 2400 hours) in a room with constant temperature (22°C) and relative humidity (55%). Twenty-four rats were assigned to 2 groups. Group 1 consisted of 13 rats that received unilateral 6-OHDA infusions; of those, 7 were later implanted on the lesioned side with hRPE-GM and 6 with gelatin microcarriers (GMs) alone. Group 2 consisted of 11 rats that received bilateral intracerebroventricular (i.c.v.) 6-OHDA infusions; of those, 7 were later implanted on the most affected side with hRPE-GM and 4 with GMs alone.

A few animals in each group were used to assess lesion severity with autoradiographic binding (see below). The majority of animals from Group 1, plus additional animals that also underwent lesioning and implantation, were used for postmortem histologic identification of surviving hRPE cells (20). The additional implanted animals that were used for early histologic time points (48 hours and 1 and 4 weeks) were not behaviorally tested because of the time-consuming nature of testing and to allow them sufficient recovery time after implantation. The results from this histologic study are presented in the companion article (20). The remaining animals from Group 2 were used in other studies.

Surgical Procedures

All surgical procedures were performed under isoflurane anesthesia (Aerrane; Baxter, Mississauga, ON, Canada). Isoflurane was set at 4% with a 60 mL/min oxygen flow for induction and 1.5% to 2% for maintenance. Atropine sulfate (Atro-Sa; 0.05 mg/kg; Rafter, Calgary, AB, Canada) was injected subcutaneously (s.c.) to decrease respiratory secretions. Rats were placed in a Kopf stereotaxic frame with the needle was rinsed once with Dulbecco’s modified Eagle’s medium. The syringe was loaded with fresh cell slurry before each implant.

A sterile Hamilton syringe with a micro-polished needle was filled with 20 µL of hRPE-GM slurry and loaded to 16 µL. The syringe was loaded with fresh cell slurry before each implant.

At each implant coordinate, the loaded Hamilton syringe was attached to the stereotaxic frame and the needle was advanced to 0.5 mm below the deepest implant coordinate (6.0 mm below the skull) to create a pocket and then brought back up to the implant site level. Using a “pulse-like” injection technique, 3 µL was injected in the first site. The needle was then slowly brought up 2 mm, and the last 3 µL was injected at the second implant site (4.0 mm below the skull). The needle was held in place for 5 minutes before being removed. GM-alone implants were performed following the same procedure.

6-Hydroxydopamine Lesions

One hour before 6-OHDA lesioning, animals received desipramine hydrochloride (25 mg/kg intraperitoneal [i.p.] injections of dissolved in sterile water; Sigma-Aldrich, Oakville, ON, Canada) to prevent noradrenergic cell damage. For unilateral lesions, 6-OHDA (10 µg/4 µL in 0.05% ascorbic acid in sterile 0.9% saline; Sigma Chemical Co., St. Louis, MO) was slowly infused (1 µL/min) in 2 sites along the right medial forebrain bundle at the following coordinates: anteroposterior (AP) −2.8, mediolateral (ML) −1.8 (from bregma), and dorsoventral (DV) 8.0; and AP −4.7, ML −1.5 (from midline) and DV: 7.9 (21). The cannula was left in place for an additional 4 minutes to allow diffusion before removal. For bilateral lesions, animals received an injection of 175 µg of 6-OHDA in 3.5 µL at the same rate, in each of the lateral ventricles, at the following coordinates: AP −0.8, ML ±1.4 (from bregma), and DV 3.8 (21).

Human Retinal Pigment Epithelial Cell Implants

Specific cell culture and cell preparation before implant are described in the companion article (20). Briefly, 1 week before implant, hRPE cells were rapidly thawed, resuspended in fresh complete medium, and then centrifuged and transferred in an unaminulated T-25 cell culture flask. To maximize cell viability, hRPE cells were grown to confluence in an incubator at 37°C before attachment to GMs and implantation. Dry GMs (40- to 60-µm diameter) were hydrated and autoclaved for sterility. The prepared hRPE cells were added to the GMs, and the mixture was placed in an incubator at 37°C overnight. Immediately before implant, cell viability was assessed using the trypan blue exclusion method (minimum required cell viability: 80%; 2,000–3,000 cells/µL). GM-alone suspensions were treated in a similar manner. All hRPE-GM and GM-alone slurry preparations were kept in an ice bath during the implantation procedure to a maximum storage time of 5 to 6 hours.

Unilaterally lesioned animals were implanted in the lesioned caudate putamen and bilaterally lesioned animals were implanted in the caudate putamen contralateral to the most affected side of the body. Animals were unilaterally implanted with 2 tracks of either 6 µL of hRPE-GM each (~12,000–15,000 hRPE cells) or an equivalent volume of GM alone, 10 weeks after the 6-OHDA lesion. Coordinates for Track 1 were AP +1.6 and ML −2.5 (from bregma) and for Track 2 were AP −0.4 and ML −3.5 (from bregma) (20). In each track, hRPE-GM and GM-alone implants were injected at 6.0 and 4.0 mm below the skull.

A sterile Hamilton syringe with a micro-polished needle was rinsed once with Dulbecco’s modified Eagle’s medium and then with Hanks’ balanced salt solution and was filled with 20 µL of Hanks’ balanced salt solution. The first 10 µL was disposed of. The needle was lowered into the Eppendorf tube containing the hRPE-GM slurry and loaded to 16 µL. The syringe was loaded with fresh cell slurry before each implant.

At each implant coordinate, the loaded Hamilton syringe was attached to the stereotaxic frame and the needle was advanced to 0.5 mm below the deepest implant coordinate (6.0 mm below the skull) to create a pocket and then brought back up to the implant site level. Using a “pulse-like” injection technique, 3 µL was injected in the first site. The needle was then slowly brought up 2 mm, and the last 3 µL was injected at the second implant site (4.0 mm below the skull). The needle was held in place for 5 minutes before being removed. GM-alone implants were performed following the same procedure.

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RPE Implants Improve Deficits in Rat PD Model

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Behavioral Testing

Testing was performed within the first 6 hours of the dark part of the light/dark cycle (12:00–18:00) when animals were more active. Unilaterally lesioned rats (Group 1) were tested before lesioning, at 10 weeks after lesioning (before implant), and at 8–10 and 18–20 weeks after implantation. Bilaterally lesioned rats (Group 2) were tested before lesioning, 10 weeks after lesioning (before implant), and at 2–4 and 8–10 weeks after implantation. Bilaterally lesioned animals were part of our initial methodologic development study and were killed at 10 weeks after implantation. Because of the significant amount of postoperative care needed (after lesioning) to maintain bilaterally lesioned animals, we elected to alter the study design and performed the remainder of the study in unilaterally lesioned animals (Group 1). In addition, the preliminary analysis of the original bilaterally lesioned Group 2 led us to extend the survival time of the unilaterally lesioned Group 1 to 20 weeks after implantation. For data comparison between the 2 groups, behavioral testing was also performed at 10 weeks after implantation in the unilaterally lesioned animals.

Forelimb Function: Forelimb Use Asymmetry Test (Cylinder Test)

The forelimb use asymmetry test was performed as described previously (17). Testing was performed under red lighting. Rats were placed in the cylinder and exploratory motion was videotaped for 3 to 5 minutes per session for a total of 2 to 4 sessions (maximum 2 per week on nonconsecutive days) to acquire a sufficient number of movements. Scoring was performed at a later time by an experimenter blind to the animals’ condition. Each particular forelimb movement (contralateral, ipsilateral, or both) was expressed in terms of percentage of use (mean ± SEM) of that limb relative to the total number of movements (i.e., percent contralateral forelimb use = 100 × [contraindependent movements]/[contralateral + ipsilateral + both]). A single score indicative of forelimb use asymmetry was obtained by calculating the difference between contralateral (impaired/implanted) and ipsilateral (nonimpaired/nonimplanted) percent use; thus, the greater the negative score, the greater the asymmetry and vice versa.

Hindlimb Function: Ledged Tapered Beam Walking Test

The hindlimb sensorimotor function test has been described in detail previously (18, 22). Briefly, the apparatus consists of a tapered beam with underhanging ledges on each side, which permit the measurement of foot faults (steps on the ledge) without the risk of falling for the rat. The beam is divided into 3 sections: wide, medium, and narrow, which represent progressive levels of difficulty. The addition of the ledges reduces the learned compensation mechanisms reported in early studies in PD and stroke rodent models. Rats with unilateral 6-OHDA lesions use the contralateral ledge as a crutch, whereas bilaterally lesioned animals may even straddle the beam. Training was done only once on the day before the first testing day (prelesion) and consisted of 10 trials, with the animals walking along the beam into their darkened home cage. Testing was done with the lights on during the dark part of the animals’ light/dark cycle. Five consecutive tests corresponded to one complete test. All trials were videotaped and scored at a later date by an investigator blind to the experimental groups. The rats’ performance was calculated as the slip percentage of the impaired hindlimb (contralateral to the implant and/or the lesion): (total number of slips [foot faults]/total number of steps) × 100 over the 5 trials for each beam section.

Postmortem Autoradiography

Lesion severity was determined in a small sample of each group using [3H]WIN 35,428 binding to the dopamine transporter in the striatum. For unilaterally lesioned animals, the percent lesion was measured with respect to the nonlesioned hemisphere, whereas with bilaterally lesioned animals, the percent lesion was determined with respect to a vehicle-infused sham lesion group. The animals were killed by decapitation, and their brains were removed and frozen in isopentane cooled to −70°C in dry ice. The brains were then stored at −80°C until sectioning. Binding to the dopamine transporter using [3H]WIN 35,428 was performed on 20-μm sections as described previously using phosphor imaging autoradiography (23). Striatal binding was measured on at least 6 sections per animal, and after subtraction of nonspecific binding, the average specific binding data for each striatum were converted to percent lesion.

Data Analysis

One-way repeated-measures analysis of variance (ANOVA) with planned comparisons was conducted to compare postimplant behavioral test performance to preimplant test performance. When significant F values were found, planned comparisons using the Bonferroni comparison test were used to determine implant effect (postlesion compared with each of the 2 postimplant time points) with p < 0.05 considered to be statistically significant.

Prelesion data were not included in the repeated ANOVA analysis as the main goal of this study was to determine the hRPE-GM implant effects on a lesioned striatum. When compared with the lesion effect, the implant effects would be expected to be discrete and overshadowed by the lesion effect. By analogy, data from the preclinical condition are rarely included in the analysis of therapeutic effects in clinical studies. The lesion effect was, however, evaluated separately using a t-test between prelesion and preimplant data.

RESULTS

All unilaterally lesioned animals recovered well after 6-OHDA lesions. There were no signs of morbidity, the mortality rate was low (<10%), and minimal postoperative care was needed.

Bilaterally lesioned animals demonstrated different recovery characteristics. There was increased morbidity and the mortality rate was higher (20%–25%) compared with the unilateral lesion group. Bilaterally lesioned rats
needed considerable postoperative care; each rat was given daily subcutaneous fluids, and the most severely affected rats were manually (tube) fed with a high-protein liquid diet. However, there were no differences in mortality rate in unilaterally and bilaterally lesioned animals in response to the implant. The shortened experimental period of the bilaterally lesioned group (8–10 weeks versus 18–20 weeks in the unilaterally lesioned group) was due to the initial study design and not to changes in the mortality rate as a result of the implant.

Behavioral Effects of Gelatin Microcarrier-Attached Human Retinal Pigment Epithelial Cell Implants in a Unilateral Rat Model of Parkinson Disease

Forelimb Function

Before the implant, both hRPE-GM and GM-alone implanted animals displayed a significant lesion effect in the cylinder test demonstrated by a decrease in contralateral forelimb use (p < 0.001), an increase in ipsilateral forelimb use (p < 0.002), and a decrease in simultaneous (both) limb use (p < 0.003) during vertical exploration (Fig. 1, ***).

A repeated-measures ANOVA revealed a significant implant effect on the mean percent use of the contralateral forelimb in hRPE-GM implanted animals (F_{2,12} = 4.64, p = 0.03) but no effect in the GM-alone implanted group (F_{2,10} = 0.81, p = 0.47). Post-hoc planned comparisons showed a significant increase in mean percent use of the contralateral forelimb in the hRPE-GM group 18–20 weeks postimplant (p < 0.05, Fig. 1A, *), but no significant differences were found at 8–10 weeks postimplant or between prelesion and postimplant scores.

The hRPE-GM group showed a trend in improvement of the mean percentage (100 × [postimplant % use – postlesion % use]/[prelesion % use – postlesion % use]) of simultaneous forelimb use at 8–10 weeks (7.20 ± 6.77%) and 18–20 weeks (35.57 ± 14.88%) postimplant. There was no improvement for GM-alone implanted animals in their mean simultaneous forelimb use: −11.77 ± 7.08% and −4.37 ± 10.63% at 8–10 and 18–20 weeks, respectively (Fig. 1B).

FIGURE 1. Effects of gelatin microcarrier-attached human retinal pigment epithelial cells (hRPE-GM) (n = 7) (A) and gelatin microcarrier (GM)-alone implants (n = 6) (B) on forelimb use in rats with severe unilateral 6-hydroxydopamine lesion. A significant increase in the independent use of the contralateral forelimb was seen at 18–20 weeks postimplant. No behavioral recovery was seen in the GM-alone group. Bars represent mean (±SEM) percentage use during vertical exploration in the cylinder. *, Significantly different at p < 0.05. ***, Significantly different at p < 0.001.

FIGURE 2. Effects of gelatin microcarrier-attached human retinal pigment epithelial cells (hRPE-GM) implants (n = 7) (A) and gelatin microcarrier (GM)-alone implants (n = 6) (B) on hindlimb function after severe unilateral 6-hydroxydopamine lesion on the ledged tapered beam walking test. There was a trend in improvement (reduced number of errors) at all levels of the ledged beam; however, a significant decrease in errors was found only at the narrow section at 18–20 weeks postimplant. Bars represent mean (±SEM) percentage of errors/steps. *, Significantly different at p < 0.05. **, Significantly different at p < 0.01.
However, these scores did not reach statistical significance. There were no differences in ipsilateral forelimb use in either the hRPE-GM or GM-alone group.

Forelimb use asymmetry scores increased (toward the negative side) after unilateral lesioning in all animals in favor of the unimpaired (ipsilateral) forelimb ($p < 0.001$). Forelimb use asymmetry scores went from $-14.22 \pm 11.22\%$ prelesion to $-9.38 \pm 4.61\%$ postlesion. There was a progressive decrease in forelimb use asymmetry after implantation in hRPE-GM implanted animals (scores became less negative), a reduction that did not reach statistical significance at 8–10 weeks (6.96\% mean improvement from baseline) but was statistically significant at 18–20 weeks postimplant (19.98\% mean improvement from baseline, $F_{2,12} = 4.68, p = 0.031$). GM-alone implanted animals did not show significant improvement between the postlesion and any of the postimplant time points (3.0\% mean improvement from baseline at 8–10 weeks and 4.49\% at 18–20 weeks).

### Hindlimb Function

All animals displayed a strong lesion effect as indicated by a significant increase in the mean percentage of errors/steps of the contralateral hindlimb in the narrow section of the beam after unilateral lesioning ($p < 0.002$), but there was no significant lesion effect detected in the wide or the medium beam sections (Fig. 2). There was a progressive decrease in the mean percentage of errors/steps with the contralateral limb in all of the sections of the tapered beam postimplant in the hRPE-GM group (Fig. 2A). This decrease in limb errors was statistically significant at 18 to 20 weeks postimplant, when animals made significantly less errors/steps with the contralateral hindlimb in the narrow section, compared with postlesion values ($p < 0.05$) (Fig. 2A, *). No statistically significant differences were found between the postlesion and the earlier (8–10-week) time point. GM-alone implanted animals did not show significant changes on the contralateral hindlimb percentage of errors/steps in any of the beam sections postimplant (Fig. 2B).

The percentage of improvement from baseline (prelesion) was calculated for both hRPE-GM and GM-alone implanted rats, using the contralateral hindlimb percentage of errors/steps for each beam section and time point in the following formula: $100 \times (\text{postlesion} - \text{postimplant})/(\text{postlesion} - \text{prelesion})$ (Table).

<table>
<thead>
<tr>
<th>Beam Section</th>
<th>Unilateral 6-OHDA + Unilateral Implant</th>
<th>Bilateral 6-OHDA + Unilateral Implant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8–10 weeks</td>
<td>18–20 weeks</td>
</tr>
<tr>
<td>hRPE-GM Wide</td>
<td>53.92</td>
<td>80.39</td>
</tr>
<tr>
<td>Medium</td>
<td>47.53</td>
<td>62.99</td>
</tr>
<tr>
<td>Narrow</td>
<td>1.14</td>
<td>48.86</td>
</tr>
<tr>
<td>GM alone Wide</td>
<td>$-13.50$</td>
<td>18.41</td>
</tr>
<tr>
<td>Medium</td>
<td>4.42</td>
<td>$-17.78$</td>
</tr>
<tr>
<td>Narrow</td>
<td>20.22</td>
<td>$-6.73$</td>
</tr>
</tbody>
</table>

100 × (postlesion – postimplant)/(postlesion – prelesion).

hRPE-GM, gelatin microcarrier-attached human retinal pigment epithelial cells; GM, gelatin microcarrier; 6-OHDA, 6-hydroxydopamine.
Behavioral Effects of Gelatin Microcarrier-Attached Human Retinal Pigment Epithelial Cell Implants in a Bilateral Rat Model of PD Forelimb Function

hRPE-GM implanted animals showed an implant effect on the mean percent independent use of the contralateral forelimb ($F_{2,12} = 3.77$, $p = 0.05$), but not of the ipsilateral forelimb ($F_{2,12} = 0.983$, $p = 0.40$). Post-hoc planned comparisons detected no significant difference in contralateral forelimb use 2–4 weeks postimplant but a significant increase in contralateral forelimb use 8–10 weeks postimplant compared with postlesion values ($p < 0.05$) (Fig. 3A, *). However, there was no significant implant effect on the independent use of the contralateral forelimb in GM-alone implanted animals ($F_{2,6} = 0.15$, $p = 0.86$) or of the ipsilateral forelimb ($F_{2,6} = 0.04$, $p = 0.96$) (Fig. 3B).

There was also an implant effect on forelimb use asymmetry scores in hRPE-GM implanted animals ($F_{2,6} = 3.84$, $p = 0.05$) but not in GM-alone implanted animals ($F_{2,6} = 0.04$, $p = 0.96$). In hRPE-GM implanted animals, there was a gradual shift in forelimb use asymmetry postimplant in favor of the contralateral forelimb that did not reach statistical significance at 2–4 weeks but became significant at 8–10 weeks postimplant ($p < 0.05$) compared to postlesion values. Forelimb use asymmetry scores in hRPE-GM implanted animals at the 4 time points during the experiment were prelesion, 6.95 ± 7.88%; postlesion, −30.24 ± 13.74%; 2–4 weeks postimplant, −17.57 ± 14.86%; and 8–10 weeks postimplant, 8.4 ± 19.56%. Corresponding values for the GM-alone implanted animals were prelesion, 16.64 ± 1.01%; postlesion, 10.33 ± 23%; 2–4 weeks post-implant, 13.72 ± 24.21%; and 8–10 weeks postimplant, 10.42 ± 33.89%.

Hindlimb Function

After i.c.v. 6-OHDA, bilaterally lesioned animals showed a moderate increase in the percentage of errors/steps with both hindlimbs, mainly in the medium and narrow sections of the tapered beam. hRPE-GM implanted animals showed a progressive decrease in the percentage of errors/steps with both limbs postimplant, but the decrease was predominantly seen in the contralateral hindlimb, an effect that reached statistical significance 8–10 weeks postimplant ($p < 0.05$) (Fig. 4A, *). GM-alone implanted animals did not show a decrease in the contralateral hindlimb percentage of errors/steps (Fig. 4B). The percent improvement from baseline (prelesion) is shown in the Table.

It should be noted that in both unilaterally and bilaterally lesioned animals, there was only partial behavioral recovery postimplant; that is, the mean motor performance of hRPE-GM implanted animals did not return to prelesion levels in either behavioral test.

Postmortem Analysis of Lesion Severity

In unilaterally lesioned animals, lesion severity was >97%, whereas in bilaterally lesioned animals, binding to the dopamine transporter was reduced from 60% to 50% bilaterally in the dorsal striatum, following a medial-lateral gradient (Fig. 5).

DISCUSSION

The main purpose of this study was to assess the behavioral effects of hRPE-GM implants on motor deficits in a characterized rat model of PD. Using validated tests of motor function sensitive to striatal DA depletions, we demonstrated in the present study demonstrated that 1) hRPE-GM implants in rats with a severe unilateral nigrostriatal lesion reduced lesion-induced asymmetry of forelimb
use and decreased lesion-induced hindlimb deficits and 2) hRPE-GM implants in rats with a moderate bilateral nigrostriatal lesion increased the independent use of the forelimb contralateral to the implant, reversed the lesion-induced asymmetry of forelimb use, and decreased lesion-induced hindlimb deficits of the hindlimb contralateral to the implant. These results are in agreement with initial data in patients with PD (14), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP)-treated monkeys (16), and rodents (11).

Striatal implantation of hRPE-GM has been shown to reduce apomorphine-induced turning in a unilateral 6-OHDA rat model of PD while provoking a minimal host immune response (11). However, when one is evaluating the effects of a potential new therapy, care must be taken that the testing itself does not affect the results (i.e. the potential interactions between the drug and the therapy to be tested have to be taken into consideration). For example, hRPE cells express DA D2 receptors and the effects of repeated apomorphine stimulation on hRPE cells are unknown and could either enhance the response, overestimating its positive effects, or diminish the response, underestimating its efficacy. Thus, in recent years there has been a push to complement drug-induced studies with adequate sensorimotor behavioral tests to provide greater interpretability of the efficacy of experimental therapies (24). Two convenient sensorimotor tests that were sensitive to unilateral and bilateral lesions of the nigrostriatal pathway in rodent models of PD were used in this study: the forelimb use asymmetry test and the ledged tapered beam walking test (17, 18, 22). These tests do not make use of aversive motivation or food deprivation and are not sensitive to the effect of practice or weight gain when performed repeatedly over extended periods of time (25). Using tests of forelimb and hindlimb motor function allows broader and more complementary behavioral assessment. Additionally, in our hands, animals with DA nigrostriatal depletions have not shown signs of spontaneous recovery in these tests when tested multiple times and for up to 5 months after 6-OHDA infusion (25).

Using a 6-OHDA unilateral lesion model allowed us to study the effects of the implants in a severely (>97%) lesioned DA nigrostriatal system, whereas the use of the bilateral model obtained by i.c.v. infusion of 6-OHDA allowed us to study the effects of the implants in a moderately DA-depleted (50%–60%) rat. It is not feasible to produce a bilaterally severely lesioned model as the majority of the rats die within days of 6-OHDA administration. Alternatively, in our hands, administering a reduced dose of 6-OHDA in the substantia nigra pars compacta/medial forebrain bundle creates widely variable lesion severity. The bilateral i.c.v. administration of 6-OHDA produced a stable, moderate, and reproducible lesion. This technique presented the added advantage that the motor improvements in the side contralateral to the implant could be compared to the improvement (or lack thereof) in the ipsilateral side.

One should note that the improvement in motor function after severe (>97% striatal DA depletion) unilateral 6-OHDA lesion in the hRPE-GM implanted group is unlikely to be explained by the intrinsic variability of the tests or induced by multiple test exposure or practice. We performed an earlier study of the stability of the tests in normal and unilaterally 6-OHDA lesioned rats (25) and found that the performance of rats in the 2 tests does not improve in the performance up to 20 weeks postlesion. In addition, to ensure sufficient reliability and sensitivity of the forelimb asymmetry test, rats implanted with hRPE-GM or GM-alone were administered 4 trials of the cylinder test to constitute one complete test at all time points after lesion. This number of trials was necessary to ensure that lesioned rats, which have some degree of hypokinesia and are less active than normal animals of the same age and weight range, had the opportunity to make more movements and thus obtain a relatively constant number of movements per complete test between the normal and lesioned conditions.

The use of animals with both moderate and severe lesions allowed us to make some observations that deserve to be discussed. One is that the timing of the recovery after implant appeared to be dependent on the severity of the original motor impairments and hence striatal DA loss. Moderately lesioned rats showed significant improvements by 10 weeks postimplant, whereas the improvement in animals with severe impairments did not reach significance before 20 weeks postimplant. A similar phenomenon was observed in MPTP-treated primates receiving unilateral implants (D.J. Doudet et al, unpublished observations, 2006). If, indeed, the principal mechanism of action of hRPE cells is the release of L-dopa in the striatum, the degree and speed of recovery is likely to be dependent on the number of remaining DA terminals in the vicinity of the implants. The L-dopa hypothesis recently gained some support with in vivo evidence of increased fluoro-dopa uptake in hRPE-implanted striatum of our nonhuman primates (16). In addition, in the same positron emission tomography study there was a decrease in raclopride binding in the implanted striatum. Raclopride, a specific tracer of the DA D2 receptor, was used in this instance as a surrogate marker of DA synaptic release. However, L-dopa production may not be the only mechanism involved in behavioral improvement.

Pharmacologic validation of the limb use asymmetry test demonstrated that spontaneous forelimb use in 6-OHDA-lesioned rats was sensitive not only to the initial beneficial effect of oral L-dopa treatment (26) but also to its delayed side effects (27). That is, unilaterally 6-OHDA-lesioned rats improved soon after L-dopa therapy was administrated, but test performance levels declined when drug-induced abnormal involuntary movements appeared. L-Dopa treatment administrated i.p. also acutely improves hindlimb motor deficits in hemiparkinsonian rats on the ledged tapered beam walking test (D.J. Doudet and I.L. Cepeda, unpublished observations, 2005).

Indeed, although the role of hRPE-produced pigment (melanin) in the eye remains unclear, it is known to serve as a free radical stabilizer and as an agent that can bind toxins. The hRPE also contains the antioxidant enzymes superoxide dismutase and catalase, which minimize the formation of free radicals that can damage lipid membranes, processes that
have been implicated in cell damage in PD (28). hRPE cells also play a very active role in repair processes through the secretion of growth factors and immunologic interaction (29). hRPE cells have been shown to produce trophic factors in culture, such as brain-derived neurotrophic factor and glia-derived neurotrophic factor, which are known for their beneficial effects on DA neurons (30). Thus, it is not inconceivable that alternate mechanisms of action play a role in the clinical improvements noted in both allograft and xenograft situations and account for the delay in the development and stabilization of significant clinical improvement (weeks to months) in rodents, monkeys, or patients.

A second observation specific to the rodent study is that in the ledged tapered beam walking test, a task with 3 levels of difficulty, improvement was greater in the section with a lower degree of difficulty (wide part of the beam) and less at the most challenging section (narrow). This difficulty in specific recovery was observed mainly in animals with unilateral lesions that showed impairments in all 3 sections of the beam and less in the moderately lesioned rats that had little or no impairment in the wide section.

Further, although motor recovery occurred in both hindlimb and forelimb function in both animal models, these improvements were not complete. This finding is consistent with earlier reports in patients and nonhuman primates. Patients with PD continue to receive daily l-dopa therapy, albeit at lower levels in some cases (14) and a 45% to 50% improvement is reported by 24 months after implant surgery. Apart from 2 animals, who were juvenile at the time of the implant and recovered fully, none of our mature or elderly nonhuman primates have shown full recovery, and their improvement ranged between 30% and 60% after 2 to 3 years postimplant (D.J. Doudet, unpublished observations, 2006). However, patients and monkeys as well as rats only received unilateral implants. This raises the possibility that a bilateral implant procedure may be able to further improve clinical recovery. A blinded, placebo-controlled study with bilateral hRPE-GM implants in patients with PD is underway and should answer this question in the near future.

Although observations in 3 species suggest a relationship between the hRPE cells and clinical improvement, it is still unclear: 1) whether and how long hRPE cells survive, and 2) whether they are responsible for the improvements. Subramanian et al (11) reported finding cells resembling hRPE cells at 18 weeks postimplant and that these could be stained by tyrosine hydroxylase immunohistochemistry. However, tyrosine hydroxylase is not an hRPE-specific marker, and the question of cell survival is still open. Our recent studies have addressed this important topic through the use of specific markers to identify hRPE cells postimplant, and our companion studies using immunohistochemistry and electron microscopy suggest that, once implanted in the rat striatum in the absence of immunosuppression, hRPE cells survive (20). We are in the process of performing a combined behavioral evaluation/stereologic analysis to address the relationship between improvement and hRPE cell number.

In summary, hRPE cells appear to be a suitable cell therapy option for transplantation in patients with PD. hRPE cells produce the DA precursor L-dopa, as part of the metabolic pathway leading to eumelanin synthesis (7), metabolizing tyrosine into L-dopa through the action of tyrosinase (8, 9, 31), the rate-limiting enzyme in melanin biosynthesis and an enzyme with tyrosine hydroxylase-like activity (10, 32, 33). hRPE cells can be isolated from fetal or neonatal eyes obtained from eye banks, screened and expanded in culture in quantities sufficient to allow implant in multiple patients from a single donor. Being anchorage-dependent cells, hRPE cells show improved survival when attached to biocompatible GM even in the absence of systemic immunosuppression in allograft or xenograft conditions. In recent months, striatal hRPE-GM implants have been shown to improve parkinsonian motor deficits in patients with PD (5, 13, 14), in monkeys with MPTP-induced parkinsonian (16), and in 6-OHDA-lesioned rodents (present study and Reference 11). Together with the findings of our histologic work (20), these results confirm the efficacy of implantation of cultured hRPE cells as a promising therapeutic option for many of the 6.3 million patients suffering from PD all over the world.

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