CONTINUED PRESENCE OF INTRASTRIATAL BUT NOT INTRAVENTRICULAR POLYMER-ENCAPSULATED PC12 CELLS IS REQUIRED FOR ALLEVIATION OF BEHAVIORAL DEFICITS IN PARKINSONIAN RODENTS

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder that is characterized by a unique profile of behavioral and neurobiological symptoms that result from the degeneration of dopaminergic neurons in the substantia nigra (6,20,21,24). The marked loss of dopaminergic neurons has led investigators to try to replace lost dopamine by grafting dopaminergic neurons adjacent to or directly into the denervated striatum. Studies have demonstrated that in both rodents and nonhuman primates, grafting of dopaminergic tissue alleviates the behavioral, neurochemical, and anatomical consequences of damage to the nigrostriatal system (5,8-10,15-17,22). Recent clinical studies have extended these observations to individuals afflicted with PD (4,18,19,25-29).

In the majority of studies, tissue has been grafted directly into the striatum, although it has also been suggested that placement of catecholamine-producing tissue/cells into the adjacent lateral ventricle has therapeutic potential for PD. The rationale behind this approach is to deliver L-dopa, dopamine, and other catecholamines to the striatum and the associated striatal dopamine receptors from the transplanted tissue via passive diffusion. To date, few studies have systematically evaluated the most appropriate location for grafting catecholaminergic cells. The inherent problems of placing tissue/cells within the ventricular system without subsequent movement of the graft or damaging the caudate when attaching the graft has made this comparison difficult. One method of making this comparison would be to immobilize catecholamine-producing cells within a...
polymeric device prior to implantation within either the striatum or the adjacent lateral ventricle (11). Previous results have demonstrated that polymer-encapsulated PC12 cells survive for extended periods of time following implantation into both rodent and primate models of PD while continuing to express tyrosine hydroxylase (TH) and secrete catecholamines (1-3). Moreover, encapsulated PC12 cells ameliorate drug-induced motor asymmetries in lesioned rats (25,32,33,35), reduce the impairments in coordination and balance in aged rats (13,14), and induce recovery of motor function in MPTP-lesioned primates (3,23). Given the contained nature of encapsulated cells, this provides an ideal system for examining the therapeutic potential of implanted catecholamine-producing cells within or adjacent to the dopamine-depleted striatum.

The present study was conducted to determine 1) if placement of encapsulated PC12 cell-loaded devices into the lateral ventricle of 6-OHDA-treated rats is as effective as intrastriatal implants at reducing apomorphine-induced rotational behavior, and 2) if the survival of PC12 cells is differentially affected by the implant site.

MATERIALS AND METHODS

Subjects
Adult male Sprague-Dawley rats (Taconic, Germantown, NY), approximately 2–3 mo old and weighing approximately 300 g, were used in the present studies. The animals were housed in groups of three to four in a temperature- and humidity-controlled colony room that was maintained on a 12 L:12 D cycle with lights on at 0700 h. Food and water were available on an ad lib basis.

Surgical Procedures
Immediately prior to surgery, rats were anesthetized with an intramuscular injection of a ketamine, xylazine, and acepromazine mixture (33 mg/1.7 mg/10 mg/mL) and positioned in a Kopf stereotaxic instrument (13). A sagittal incision was made in the scalp and a hole drilled for the unilateral injection of 6-OHDA into the substantia nigra. A total of 12 µg of 6-OHDA (6.0 µL of 2.0 µg/µL dissolved in 0.9% saline containing 0.2% ascorbic acid) was infused at a rate of 1 µL/min and allowed to diffuse for an additional 5 min prior to the removal of the infusion needle. Coordinates for infusion were 4.2 mm posterior to bregma, 1.0 mm lateral to the sagittal suture, and 7.4 mm ventral from the cortical surface (30).

Six weeks following 6-OHDA infusions, rats were again anesthetized and a single hole drilled for the unilateral placement of the polymer capsules into the striatum or lateral ventricle (12,13). The stereotaxic coordinates for implantation into the striatum were: 0.5 mm anterior to bregma, 3.8 mm lateral to the sagittal suture, and 7.5 mm below the cortical surface, and the coordinates for implantation into the lateral ventricle were: 0.5 mm anterior to bregma, 1.5 mm lateral to the sagittal suture, and 7.5 mm below the cortical surface. This resulted in the formation of six experimental groups: 1) 6-OHDA lesion only (lesion only, n = 8), 2) 6-OHDA lesion plus PC12 cells implanted into the striatum (lesion/PC12-striatum, n = 8), 3) 6-OHDA lesion plus PC12 cells implanted into the lateral ventricle (lesion/PC12-ventricle, n = 8), 4) 6-OHDA lesion plus empty capsules implanted into the striatum (lesion/empty-striatum, n = 8), 5) 6-OHDA lesion plus empty capsules implanted into the lateral ventricle (lesion/empty-ventricle, n = 6), and 6) 6-OHDA lesion plus PC12 cells implanted temporarily into the striatum (removed 4 wk following implantation and 2 wk prior to the final rotenometry test, see below) (lesion/PC12-temporary striatum, n = 8). Animals receiving temporary striatal implants had their devices removed after 4 wk. These animals were anesthetized and placed into the stereotaxic instrument. A craniotomy was performed over the implantation site and the dural scar surrounding the implant site excised. The cortical surface was cut to expose the underlying capsule that was retrieved with a pair of Dumont (#5) forceps. Following removal, these animals were allowed to recover for an additional 1-wk period.

Cell Culture and Encapsulation
PC12 cells were maintained in vitro and harvested prior to encapsulation as previously described (12,13). Individual capsules (600 ± 30 µm in diameter and 6 ± 0.5 mm in length) were fabricated as previously described (7,12,13,25). Briefly, hollow fiber membranes were fabricated with a phase-inversion technique using poly (acrylonitrile-co-vinyl chloride) (P(AN-VC). These fibers have a dense inner permselective barrier on the luminal surface and a large macrovoid trabecular wall structure. Individual capsules were loaded with 25,000–30,000 PC12 cells mixed in a chitosan matrix as previously described (12) and maintained in Neurobasal medium supplemented with B27 (Gibco, Inc. Grand Island, NY) for 4–5 days prior to implantation. Controls consisted of devices that contained the matrix (chitosan) alone. Prior to implantation, the control and PC12 cell-loaded devices were assayed to quantitate L-dopa and dopamine release (see below).

Neurochemical Analysis
Three days following the encapsulation procedure, free-floating capsules were washed twice with 1 mL of HBSS HEPES-buffered saline (containing 10 µM ascorbate) to remove residual culture medium. Sampling con-
sisted of a 30-min incubation (basal release) in 250 μL HEPES-buffered HBSS followed immediately by a 15-min incubation in 250 μL HEPES-buffered HBSS containing 56 mM potassium (stimulated release). All samples were protected from oxidation by the rapid addition of a citrate-reducing acidified buffer (CRAB) and yielded a stable sample preparation in 10 mM citric acid, 20 μM sodium metabisulfate, and 0.1 N perchloric acid.

Following device retrieval from the host brain, the devices were placed in 1 mL of phosphate-buffered HBSS for approximately 30 min. The phosphate-buffered HBSS was removed and 1 mL of HEPES HBSS was placed on the devices for an additional 30 min for basal and 15 min stimulated catecholamine analysis as described above. After the catecholamine assay was completed the devices were placed in 4% paraformaldehyde and submitted for morphologic analysis (see below).

Behavioral Testing

From 2–6 wk after being infused with 6-OHDA, rats were subcutaneously injected once each week with apomorphine (0.25 mg/kg the first week and 0.05 mg/kg thereafter) and 360 degree rotations were quantified by monitoring the rats for 30 min in automated rotometers (Rotoscan Rotometer, Omnitech, Inc., Columbus, OH.) Only those rats which exhibited at least 200 complete contralateral turns during the 30-min test session were included in the subsequent behavioral analysis. Animals were tested 2, 4, and 6 wk following implantation with either control or PC12 cell-containing devices.

Histology

At the conclusion of behavioral testing (6 wk), the control and PC12 cell-loaded devices were removed from the remaining animals. Immediately thereafter, all animals were transcardially perfused using a peristaltic pump, with saline (0.9%) followed by neutral buffered paraformaldehyde (4.0%).Brains were removed 30 min following fixation, placed in paraformaldehyde for 2 h followed by 20% buffered sucrose for 24–48 h. The brains were blocked, quick frozen in dry ice, and coronally sectioned through the striatal implants on a cryocut and immediately mounted on gelatin-subbed slides. Mounted slides were immunoreacted with antisera directed against TH and GFAP as previously described (13,23). Adjacent sections were stained for hematoxylin and eosin (H+E).

For analysis of the retrieved devices, capsules were fixed in a 4% paraformaldehyde solution, rinsed in PBS and dehydrated up to 95% ethanol. A 1:1 solution of glycol methacrylate (Historesin, Reichert-Jung, Cambridge Instruments) was then added to the devices for 1 h. Pure infiltration solution replaced the 1:1 mixture and remained for a minimum of 2 h. The devices were then rinsed with the embedding solution, transferred to flat molds, and embedded in glycol methacrylate. Sections 5 μm thick were sectioned (Reichert-Jung, Supercut microtome 2065), mounted on glass slides and stained with H+E.

Data Analysis

Rotometry data was analyzed with a two-way repeated measures ANOVA (31). The preimplant and postexplant values were analyzed separately. Planned analysis failed to detect differences in behavior at any time during the experiment among the different control subgroups. Accordingly, the animals in the lesion only, lesion/empty-striatum, and lesion/empty-ventricle groups were combined into one group (designated as Controls) to facilitate statistical and graphic comparisons. Secretion of evoked L-dopa and dopamine from the devices was analyzed using a two-way repeated measures ANOVA. Device output of basal L-dopa, DOPAC, dopamine, and evoked DOPAC was analyzed using a one-way ANOVA.

Fig. 1. Apomorphine-induced rotations. The number of contralateral rotations was the same for all groups before surgery. After surgery, the number of apomorphine-induced contralateral rotations was substantially reduced, but only in rats implanted with PC12 cell-loaded devices in the striatum, and only as long as the devices remained implanted.
RESULTS

Rotometry

Prior to implantation, there were no significant differences between treatment groups with respect to the number of apomorphine-induced rotations (Fig. 1). The main effect for groups was not statistically significant, $F(3, 42) = 0.25, p = 0.86$, and the trial x groups interaction was not statistically significant, $F(5, 210) = 0.69, p = 0.79$. After being implanted with devices, the number of apomorphine-induced rotations was significantly reduced in the two groups with encapsulated PC12 cells implanted into the striatum but not in the group receiving PC12 cells into the lateral ventricle. Implantation of control matrix-containing devices into either the striatum or lateral ventricles had no effect on rotations. The main effect for Groups was statistically significant, $F(3, 42) = 10.98, p = 0.0001$ (Fig. 1). After 4 wk of testing, the devices were removed from one of the groups (lesion/PC12-temporary striatum). The reduction in apomorphine-induced rotations did not persist after device removal, resulting in a statistically significant group x trial interaction, $F(6, 84) = 9.29, p = 0.0001$.

Neurochemistry

Basal and potassium-evoked l-dopa, dopamine, and DOPAC output was measured before implantation and after explantation. Evoked l-dopa output increased over time in vivo, the main effect for time was statistically significant, $F(1, 21) = 52.16, p = 0.001$. The degree of increase in evoked l-dopa output differed between groups with the devices in the lateral ventricles showing less of an increase than the striatally placed devices, resulting in a statistically significant group x time interaction, $F(2, 21) = 4.26, p = 0.03$ (see Fig. 2). There was no significant difference between groups with respect to basal output of l-dopa after explantation, $F(2, 21) = 1.21, p = 0.32$. The groups did differ with respect to basal DOPAC and dopamine output after explantation. Basal output of both DOPAC, $F(2, 21) = 3.97, p = 0.03$, and dopamine, $F(2, 21) = 8.45, p = 0.002$, were significantly different in those devices implanted within the ventricle relative to those implanted into the striatum (Table 1). No significant differences were seen between the groups with respect to evoked DOPAC levels, $F(2, 21) = 1.20, p = 0.32$. The control devices were also assayed for l-dopa, dopamine, and DOPAC output. No measurable release was observed preimplant, although in a few cases some residual host DOPAC was observed from explanted devices under basal assay conditions.

Histology

The control and cell-loaded devices were easily retrieved with variable degrees of host tissue adhered to the explanted devices. Low levels of host tissue were adhered to the control capsule walls, while in general, abundant host tissue was observed covering the cell-loaded devices (Fig. 3). The ventricular cell-loaded implants also revealed low levels of adhering host tissue within the lateral ventricle; however, the section of the device that extended into the overlying host cortical tissue had similar levels of adherent host tissue as those devices in the striatum. All PC12 cell-loaded capsules contained clusters of well preserved PC12 cells. Areas of cellular necrosis were observed although these were located primarily within the cores of the most densely packed cellular aggregates or within the central region of the device (Fig. 3). The distribution of the PC12 cells within the capsules was consistent in the striatal devices and extended along the dorsal/ventral extent of the capsule. There were no major differences in cell viability between those devices implanted in the lateral ventricle.

<table>
<thead>
<tr>
<th>Measure</th>
<th>Striatum</th>
<th>Temporary Striatum</th>
<th>Lateral Ventricle</th>
</tr>
</thead>
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<tr>
<td>Basal L-DOPA</td>
<td>11.7 (1.4)</td>
<td>14.5 (2.1)</td>
<td>10.6 (2.2)</td>
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<tr>
<td>Basal DOPAC*</td>
<td>14.0 (1.4)</td>
<td>12.3 (1.0)</td>
<td>17.1 (1.2)</td>
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<td>Basal dopamine*</td>
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<td>0.2 (0.1)</td>
<td>1.8 (0.5)</td>
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<td>K+–stimulated DOPAC</td>
<td>11.7 (1.4)</td>
<td>10.1 (1.0)</td>
<td>12.7 (1.0)</td>
</tr>
</tbody>
</table>

Data are expressed as pmol transmitter or metabolite/30 min incubation under basal conditions and 15 min under potassium-stimulated conditions.

*p < 0.05.
Fig. 3. Representative photomicrographs of H&E-stained GMA-embedded sections of PC12 cell-loaded devices explanted after 6 wk from (A) the striatum, and (B) the lateral ventricle. The small triangular arrows illustrate the interface between the external capsule wall and adherent host tissue. Note the abundant PC12 cells that appear viable and well distributed throughout the capsules. Also, note the abundant host tissue that is adhered to the striatal device (A), while the adherent host tissue is diminished in the ventricular implant beginning at a level corresponding to the dorsal aspect of the lateral ventricle (B). Scale bar, 100 μm.

and those implanted in the striatum. However, the number and extent of proliferation appear to be influenced by device location. The portion of the PC12 cell-loaded devices extending into the lateral ventricle have lower quantities of PC12 cells and cellular material as compared to the striatal implants.

Analysis of TH-immunostained sections demonstrated a nearly complete loss of TH-positive fibers in the lesioned striatum (data not shown). Thus, all animals used in this experiment were considered to have received effective and complete lesions. In agreement with previous studies, histological analysis demonstrated accurate placement of all empty and cell-containing devices (12,13,25,32,33). Placement of the capsules within the striatum extended from the ventral to the dorsal aspect of the striatum and through the corpus callosum to abut the cortex. The capsules placed within the lateral ventricle also abutted the cortex and extended through the corpus callosum to the ventral aspect of the lateral ventricle. The extension of the devices into the adjacent striatum and septum was minimal.

The host response to the control and ventricular devices was generally regarded as low grade, while the striatal and overlying cortical contact regions elicited a response in the intermediate range. As in previous studies, there was a relative paucity of immunoreactive GFAP positive glia that was only seen at circumscribed locations at the graft-host interface (12,13,23,25,32,33). In addition, other nonneuronal cells such as macrophages and monocytes were occasionally observed at the graft-host interface. Directly adjacent to the implant, a proliferation of small to moderate-sized blood vessels was observed in the devices eliciting an intermediate grade host response. This did not appear to be associated with the number of viable cells within the explanted capsules.

DISCUSSION

The results of the present study are consistent with previous work demonstrating that polymer-encapsulated PC12 cells release significant amounts of catecholamines and are capable of reducing apomorphine-induced rotations in 6-OHDA-lesioned rats (25,32,33,35). However, the behavioral recovery was observed only in those animals receiving intrastriatal, but not intraventricular, im-
plants of encapsulated PC12 cells. The behavioral recovery in these animals suggests that a humoral molecule (presumably dopamine or L-dopa) was able to alter the consequences of nigrostriatal damage. Several lines of evidence support this contention including 1) the continued secretion of catecholamines by explanted PC12 cells in the present and previous studies (3,12,13,25,35), 2) our previous observations that encapsulated PC12 cells remain positive for TH in vitro and in vivo (1,33), and 3) microdialysis studies demonstrating that diffusion of dopamine from encapsulated PC12 cells is associated with behavioral recovery in rodents (33).

The present results also suggest that low quantities of secreted dopamine or L-dopa from polymer capsules containing catecholamine cells may be sufficient to exert a significant behavioral effect in the denervated striatum. Previous studies have demonstrated that intraventricular implants of catecholamine-producing tissue (i.e., adrenal chromaffin tissue) are capable of reducing rotational behavior in lesioned rodents [see (16,17) for reviews]. Presumably, the close juxtaposition of these grafts to the striatum resulted in passive diffusion of dopamine and other catecholamines from the cell bodies within the graft into the host striatum. Using autoradiography, the reductions in rotation behavior in these models has also been shown to be associated with a normalization of dopamine receptor supersensitivity (15). In the present studies, placement of encapsulated dopamine and L-dopa-secreting PC12 cells into the lateral ventricle was not sufficient to reduce apomorphine-induced rotation behavior. The differences between the present findings and previous work may be attributable to differences in catecholamine concentrations and subsequent diffusional restrictions. It may be that increasing the number of polymer-encapsulated PC12 cells to raise catecholamine levels would be sufficient to exert a behavioral effect following ventricular implantation. Indeed, encapsulation allows precise packaging and quantification of cell number. By measuring the catecholamine output from individual capsules it would be possible to use devices with a range of outputs to determine a dose response curve with respect to behavioral efficacy. Nevertheless, the results of the present study suggest that intraparenchymal PC12 cell-loaded devices are more potent than intraventricular devices in reducing rotation behavior.

Removal of intrastrital devices resulted in a rapid return of rotation behavior to preimplant levels. While consistent with other rodent studies (34), these findings are in contrast to studies using encapsulated PC12 cells in hemiparkinsonian primates. Intrastriatal implants of encapsulated PC12 cells in MPTP-lesioned primates resulted in a nearly complete restoration of limb use over 6 mo (23). However, the functional recovery observed in these monkeys did not dissipate following removal of the implants making the mechanism of functional recovery unclear. The most straightforward interpretation would be that the L-dopa and/or dopamine secreted from the capsules binds to supersensitive D2 receptors within the lesioned striatum restoring dopamine tone to invoke a behavioral response. It was suggested that the lack of behavioral dissipation in the nonhuman primates might be the result of a more permanent behavioral recovery induced by additional undefined factors secreted by the PC12 cells. The results of the present study do, however, lend credence to the notion that catecholamine diffusion from the devices influences the observed behavioral recovery. Moreover, these data are in clear agreement with the observation that behavioral deficits are reinstated following removal of encapsulated polymer release rods containing dopamine in lesioned animals (33). Conceivably, these differences may be accounted for by variations between the models used to evaluate functional recovery in rodents and primates.

Prior to initiating clinical trials to evaluate the use of any transplant approach in PD the safety of the methodology should be extensively documented. Several strategies have been followed in using dopamine-secreting cells for PD, including autologous adrenal tissue, fetal brain tissue, and engineered cell lines [see (16,17,22) for reviews]. Implants of primary tissue such as adrenal medullary tissue or fetal brain cells have little risk of forming tumors, but this is not necessarily the case with engineered cell lines. In addition, expression of the desired gene product in engineered cells has generally been unstable. The limited availability of fetal and adrenal tissues may also preclude their widespread clinical use. Regardless of availability, behavioral efficacy and transplant survival is essential. Adrenal tissue autografts have provided limited efficacy in PD and have generally been associated with poor graft survival [see (16) for a review]. Cell lines, in contrast, offer the ability to establish cell banks that are free of adventitious agents, can be clonal, and can be genetically engineered to enhance secretion of the desired molecule or secrete additional potentially active molecules. Finally, enclosing cells within a polymer capsule provides an extra margin of safety by confining the cells to a discrete location, preventing tumor formation, and enabling the removal of the encapsulated cells.

REFERENCES

2. Aebischer, P.; Wahlberg, L.; Tresco, P.A.; Winn, S.R.


27. Lindvall, O.; Brundin, P.; Widner, H.; Rehncrona, S.; Gus-


