Glial cell line–derived neurotrophic factor–supplemented hibernation of fetal ventral mesencephalic neurons for transplantation in Parkinson disease: long-term storage

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Object. Investigation of fetal dopaminergic tissue transplantation is being conducted in animal models and clinical trials as a potential treatment for advanced Parkinson disease (PD). Because the availability of fetal tissue is limited, however, the duration of its storage prior to transplantation is a key practical issue. Longer storage times may enable fetal tissue obtained over several days to be pooled together for transplantation in a recipient. Glial cell line–derived neurotrophic factor (GDNF) has been shown to improve survival of stored human dopaminergic tissue prior to transplantation. The objective of this study was to evaluate GDNF-supplemented hibernation of fetal dopaminergic tissue for extended periods of 6 to 15 days.

Methods. A total of 27 rat ventral mesencephalons (VMs) were obtained in gestation Day 14 rat fetuses, and three were cultured immediately (fresh-culture control group). The remaining 24 VMs were divided sagittally along the midline to form 48 equal pieces of hemimesencephalons. Twenty-four pieces were stored with GDNF-supplemented hibernation medium for 6, 9, 12, or 15 days, and the 24 “partner” hemimesencephalons were stored in control hibernation medium for the same periods of time. Tissue was cultured for 48 hours and processed for tyrosine hydroxylase (TH) immunoreactivity and cresyl violet. Cell counts for all cultures and percentage of TH-immunoreactive cells were obtained. The percentage of TH-positive cells for the fresh control group was 6.3 ± 0.5%; that measured in cultures derived from tissue hibernated in GDNF-supplemented medium was significantly increased at 6 and 9 days posthibernation compared with the fresh-culture control group and the partner groups stored in hibernation medium only. No significant increase in percentage of TH-immunoreactive cells was observed in the 12- and 15-day hibernation groups.

Conclusions. In summary the authors found that fetal dopaminergic tissue can safely be stored up to 9 days in GDNF-supplemented hibernation medium. Furthermore the percentage of TH-immunoreactive cells is significantly increased after 6 and 9 days of storage in this medium, improving the yield of TH-positive cells prior to transplantation. These observations may have important clinical implications for collecting fetal dopaminergic cells and improving their survival after transplantation.

KEY WORDS • glial cell line–derived neurotrophic factor • neural implantation • transplantation • Parkinson disease

Tissue grafts of fetal dopaminergic neurons have been investigated in animal studies2,3,13,34–37,49 and clinical trials for use as a potential treatment for advanced PD.8–10,20,21,26,29,31–33,58 Although the results reported in clinical studies have demonstrated graft survival and reinnervation of the putamen in human hosts,8,22,23 fetal tissue graft implantation in patients with PD has not reached a level of clinical efficacy that would justify its use as a routine treatment. A major disadvantage of fetal tissue is its limited supply. With current transplant methods, only 1 to 20% of the implanted dopaminergic neurons survive. To achieve useful functional recovery following transplantation, a sufficient amount of fetal mesencephalic tissue must be obtained so that the final number of surviving grafted dopaminergic neurons would total approximately 100,000 to 150,000 cells. In practice this involves deriving tissue from three to four fetuses per side per patient. Given the limited availability of fetal tissue, this is very difficult to achieve unless tissue can be pooled from specimens collected sequentially over a number of days. Consequently, tissue storage methods that maintain cell survival over the days preceding transplantation would prove very useful.

Tissue storage strategies such as cryopreservation11,42 and cool storage12,39,43,44 help to increase tissue availability by enabling the pooling of tissue obtained in several fetuses. Cryopreservation, or freeze storage, of human VM tissue, however, has produced poor graft-related results, failing to induce functional recovery and producing poor graft volumes in the rat model of PD.11 Cool storage/hibernation at 4 to 8°C has previously been successful for periods of up to 2 to 3 days, but storage for extended peri-
ods (≥ 5 days) causes reduced graft volumes and decreased TH-positive cell populations.

To increase survival of TH-positive dopaminergic neurons in cool storage, we have supplemented our hibernation medium with GDNF. Both GDNF and a number of related factors are a newly characterized group of trophic factors related to the transforming growth factor–β superfamily that signal through a RET receptor tyrosine kinase. Glial cell–derived neurotrophic factor was initially isolated as a trophic factor for midbrain dopaminergic neurons. It has been shown to increase TH-positive cell survival and fiber outgrowth when infused directly at the graft site; additionally it protects dopaminergic cells from neurotoxins such as 6-hydroxydopamine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and 1-methyl-4-phenylpyridine.

Furthermore, GDNF has been implicated in the natural development of the nigrostriatal pathway, and it promotes expression of the typical midbrain dopaminergic neuron morphology in mesencephalon-derived progenitor cells. In our studies of GDNF, we found that cool storage in GDNF-supplemented hibernation medium improves cell survival, fiber outgrowth, and promotes faster recovery in the rat model of PD. We have also shown that GDNF supplementation of hibernation medium protects human fetal dopaminergic neurons in cool storage, and we have applied this strategy to storage of VM tissue for 6 days prior to transplantation in patients with PD.

The objective of the present study was to investigate the efficacy of cool storage of fetal VM tissue in GDNF-supplemented hibernation medium beyond 6 days without decreased dopaminergic cell viability.

**MATERIALS AND METHODS**

**Tissue Collection and Hibernation**

Ventral mesencephalons were dissected in 27 Day 14 rat fetuses. Three of these 27 VMs were placed in culture immediately as fresh controls. The remaining 24 samples were sectioned sagittally along their midlines to form 48 equal pieces of hemimesencephalons. Half of these were placed in 6-, 9-, 12-, and 15-day hibernation groups that received GDNF-supplemented hibernation medium, and the other half were placed in 6-, 9-, 12-, and 15-day hibernation groups that received hibernation medium alone. Hibernation consisted of cool storage for 6, 9, 12, or 15 days at 4°C in 500 μL of a low-sodium, phosphate-buffered, calcium-free storage medium. For GDNF-supplemented hibernation, recombinant human GDNF (1 μg/ml; Prepro Tech Inc., Rocky Hill, NJ) was added to the hibernation medium on the 1st day of hibernation.

**Cell Culture**

Ventral mesencephalon tissue was incubated in 0.1% trypsin in 0.05% DNase/DMEM for 20 minutes at 37°C and then washed in 0.05% DNase/DMEM to stop trypsin activity (trypsin, DNase, and DMEM; Sigma, Chicago, IL). The tissue was mechanically dissociated by trituration using successively smaller sterilized micropipette tips until a “chunky” cell suspension was achieved. The cell suspension was centrifuged at 300 G for 5 minutes by using a small bench-top centrifuge, and the cell pellet was subsequently resuspended in culture medium. Cell count and viability was determined using the trypan blue exclusion test. Cell suspensions were plated onto a poly–L-lysine-coated substrate (10 μg/ml; Sigma) at 200,000 cells/cm² in serum-free media consisting of DMEM/F12 (Sigma) 3:1, B27 (Gibco, Burlington, ON, Canada) 1:50 penicillin 100 U/ml, and streptomycin 100 μg/ml. All cultures were maintained at 37°C in a humified incubator with a 5% CO₂ atmosphere for 48 hours.

**Tyrosine Hydroxylase Immunohistochemistry and Cell Counts**

Immunohistochemistry for TH was conducted on 4% paraffin-parafomaldehyde fixed slides. Briefly, cell cultures were quenched in 3% H₂O₂/10% MeOH for 10 minutes, followed by blocking for 1 hour with normal swine serum in 0.3% Triton-X100. Cell cultures were incubated in primary antibody, rabbit anti–TH (1:750; Pel Freeze, Rogers, AK), overnight at room temperature. They were then incubated in swine anti–rabbit secondary antibody and avidin–biotin complex (Vector Dimension, Burlington, ON, Canada) for 1 hour each. Finally, the cultures were placed in diaminobenzidine/H₂O₂ for 5 to 10 minutes. Cell counting was performed on cells stained for TH immunoreactivity. Cell counts were made using an eyepiece grid (0.25 × 0.25 mm divided into a 10 × 10 grid), counting TH-positive cells and cresyl violet–stained non–TH–positive cells within a fixed area. Sample areas were chosen using a randomized number generator that generated (x and y) coordinates within the total culture area. All data were statistically analyzed using two-way analysis of variance and two-sample t-tests.

**RESULTS**

All groups yielded healthy-appearing cultures when examined under phase-contrast microscopy during the 48-hour culture period (Fig. 1). The percentage of TH-positive cells was increased in the GDNF-supplemented group for all hibernation periods compared with the groups stored in hibernation medium alone; however, this percentage was only statistically significantly higher in the 6- and 9-day groups (Fig. 2). This increase in percentage of TH immunoreactivity was also significantly higher than fresh (immediately cultured) controls. The percentage of TH-positive cells declined after 9 days in hibernation, becoming significantly less than that observed fresh control values in the 15-day hibernation group.

**DISCUSSION**

It is estimated that only 1 to 20% of grafted dopaminergic cells survive neural transplantation. Consequently, a large amount of cells—derived from multiple donor fetuses—is required for a transplant to produce meaningful functional outcomes. Because of the difficulties surrounding the acquisition of fetal donors and the limited supply of donors of the correct gestational age, tissue storage is desirable to allow collection over several days. Furthermore, complete microbiological screening for infectious diseases in fetal tissues is more easily accom-
plished when storage times are extended. Tissue storage methods such as freeze storage and cool storage for greater than 3 days, however, result in decreased graft volumes and decreased functional recovery in animal studies and clinical trials. We subsequently demonstrated that adding GDNF to the hibernation medium resulted in increased fiber density in the zone surrounding the graft, as well as increased functional recovery as measured by the adjusting step test in the rat model of PD. We have also observed that human VM tissue stored for 6 days in non-GDNF-supplemented hibernation medium undergoes a 31% decline in TH-positive cell populations compared with fresh controls and tissue hibernated with GDNF.

In the present study, we examined GDNF–supplemented hibernation for extended time periods of 6 to 15 days. The GDNF–supplemented hibernation promotes a significant increase of the percentage of TH immunoreactivity cells when tissue is stored up to 9 days at 4°C compared with both fresh control (immediately cultured) and non-GDNF-supplemented hibernation groups. This increase is not observed at 12 and 15 days. Our results suggest that storing fetal tissue in hibernation media for up to 9 days is beneficial, when supplemented with GDNF. This observation has important practical implications for neural transplantation because hibernation with GDNF will enhance the percentage of dopaminergic cells and allow the pooling of fetal tissue over several days, enabling a sufficient quantity of VM cells to be collected to produce meaningful functional outcomes in transplanted patients undergoing transplantation.

Although the precise molecular mechanism(s) underlying the “dopaminotrophic” effect of GDNF remains unclear, one suggested mechanism is the suppression of caspase activity. Caspases are mediators of neuronal apoptosis, and reactive oxidative stress has been demonstrated to cause apoptosis via caspase activation. Hibernation of tissue may lead to the buildup of intracellular free radicals due to ischemia, and reactive oxidative metabolites may be released from lysis of cells. Thus GDNF may prevent oxidative stress–induced apoptosis by preventing caspase activation.

It is likely that the antiapoptotic effect of GDNF involves protein synthesis via induction of nuclear transcription. Sawada and colleagues have reported that simultaneous administration of GDNF with apoptosis-inducing neurotoxins to midbrain neurons fails to rescue the neurons from the action of the toxins. This observation is in line with data obtained by our group showing that GDNF-supplemented hibernation of VM tissue prior to transplantation led to significantly increased fiber outgrowth and percentage of TH-positive cells per graft, compared with transplants of fresh VM tissue grafted with (but not stored in) GDNF. These data would appear to suggest that the antiapoptotic actions of GDNF require the de novo synthesis of proteins.

Preincubation with neurotrophic factors may rescue a population of cells that die early in hibernation or in transplanted grafts. Sauer and Brundin noted that although there was a significant decrease in TH-positive cell populations of VM hibernated for 24 hours, as determined by cell-smear immunohistochemistry, there was no difference in TH-positive cell populations in grafts of fresh tissue compared with tissue hibernated for 2 days. They pro-
posed that there exists a population of TH-positive cells destined to die within the first 24 hours in hibernation or after grafting. Preincubation with GDNF in the cell hibernation medium may rescue this particular cell population from early cell death.61

CONCLUSIONS

Glial cell line–derived neurotrophic factor has been shown to enhance VM grafts and increase cell survival in the rat model of PD, as well as prevent the decline in human fetal VM TH-positive cell survival that is typically seen beyond 3 days in cool storage. In the present study we found that exposing rat VMs to GDNF during tissue hibernation increases the percentage of TH-immunoreactive cell populations for up to 9 days following extraction from fetuses, compared with fresh (immediately cultured) control. This finding bears clinical importance because the technique of GDNF-supplemented hibernation of fetal VM cells will enable longer storage periods of graft material, a critical practical issue in neural transplantation for PD.

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GDNF-supplemented hibernation


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