Grafts of fetal dopaminergic neurons have been investigated in animal studies and clinical trials as a potential treatment for advanced Parkinson disease (PD). Although the results in clinical studies have demonstrated graft survival and reinnervation of the putamen in human hosts, fetal tissue grafting 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, because with current transplant methods, only 1 to 20% of the implanted dopaminergic neurons survive. To achieve useful functional recovery after transplantation, a sufficient amount of fetal mesencephalic tissue must be obtained so that the final number of surviving grafted dopaminergic neurons would total 100,000 to 150,000 cells. In practice this involves deriving tissue from several fetuses. Nevertheless, cryopreservation of fetal mesencephalic tissue has produced poor grafting results, failing to induce functional recovery and producing poor graft volumes in the rat model of PD. 

Although the results in clinical studies have demonstrated graft survival and reinnervation of the putamen in human hosts, fetal tissue grafting 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, because with current transplant methods, only 1 to 20% of the implanted dopaminergic neurons survive. To achieve useful functional recovery after transplantation, a sufficient amount of fetal mesencephalic tissue must be obtained so that the final number of surviving grafted dopaminergic neurons would total 100,000 to 150,000 cells. In practice this involves deriving tissue from several fetuses. Nevertheless, cryopreservation, or frozen storage, of human VM tissue has produced poor grafting results, failing to induce functional recovery and producing poor graft volumes in the rat model of PD. 

Cool storage/hibernation at 4 to 8°C has previously been successful for storage periods of up to 2 or 3 days, but storage for extended periods (5 days and longer)
Hibernation of VM cells in GDNF-supplemented medium

causes reduced graft volumes\textsuperscript{22} and decreased TH-positive cell populations.\textsuperscript{39,43}

To increase the survival of TH-positive dopaminergic neurons in cool storage, we have supplemented our hibernation medium with GDNF.\textsuperscript{1,30,32,33} This factor, along with a number of related ones, belongs to a newly characterized group of trophic factors that are related to the transforming growth factor–\(\beta\) superfamily that signal through an RET tyrosine-kinase receptor.\textsuperscript{6,16,54–56} The GDNF was initially isolated as a trophic factor for midbrain dopaminergic neurons.\textsuperscript{27} It has been shown to increase TH-positive cell survival and fiber outgrowth when infused directly at the graft site, and protects dopaminergic cells from neurotoxins such as 6-hydroxydopamine, 1-methyl-4-phenyl-2,5,6-tetrahydropyridine, and 1-methyl-4-phenylpyridinium.\textsuperscript{7,14,19,24,45,47,48} Furthermore, GDNF has been implicated in the natural development of the nigrostriatal pathway,\textsuperscript{5,51} and promotes expression of the typical midbrain dopaminergic neuron morphological features in mesencephalon-derived progenitor cells.\textsuperscript{40}

In our studies in which GDNF was used, it was demonstrated that cool storage in GDNF-supplemented hibernation medium improves cell survival and fiber outgrowth, and promotes faster recovery in the rat model of PD. We have also shown that GDNF supplementation of the 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.\textsuperscript{32,33}

The objective of this study was to investigate if cool storage of fetal VM tissue in GDNF-supplemented hibernation medium is possible beyond 6 days without decreasing dopaminergic cell viability.

Materials and Methods

Tissue Collection and Hibernation

The VMs were dissected from 27 14-day-old rat fetuses. Three of the 27 VMs were placed in culture immediately as fresh controls. The remaining 24 were sectioned sagitally along their midlines for 48 equal pieces of hemimesencephalon. 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 without GDNF. Hibernation consisted of cool storage for 6, 9, 12, or 15 days at 4 \(^\circ\)C in 500 \(\mu\)l of a low-sodium, phosphate-buffered, calcium-free storage medium.\textsuperscript{1,10} For GDNF-supplemented hibernation, recombinant human GDNF (1 \(\mu\)g/ml; Prepro Tech, Inc., Rocky Hill, NJ) was added to the hibernation medium on the 1st day of hibernation.

Cell Culture

The VM tissue was incubated in 0.1% trypsin in 0.05% DNase/DMEM for 20 minutes at 37 \(^\circ\)C and then washed in 0.05% DNase/DMEM to stop trypsin activity. The trypsin, DNase, and DMEM were obtained from Sigma, Chicago, IL. The tissue was mechanically dissociated with trituration by using successively smaller sterilized micropipette tips until a “chunky” cell suspension was achieved. This cell suspension was centrifuged at 300 G for 5 minutes in a small bench-top centrifuge, and the cell pellet was subsequently resuspended in culture medium. The cell count and viability were determined using the trypan blue exclusion test. Cell suspensions were plated onto a poly-L-lysine–coated substrate (10 \(\mu\)g/ml; Sigma) at 200,000 cells/cm\(^2\) in serum-free media consisting of 3:1 DMEM/F12 (Sigma); 1:50 B27 (Gibco, Burlington, ON, Canada); 100 U/ml pen-

icillin; and 100 \(\mu\)g/ml streptomycin. All cultures were maintained at 37 \(^\circ\)C in a humidified incubator with a 5% CO\(_2\) atmosphere for 48 hours.

Immunohistochemical Findings for TH and Cell Counts

Immunohistochemical studies for TH were performed on 4% paraformaldehyde-fixed slides. Briefly, cell cultures were quenched in 3% H\(_2\)O\(_2\)/10% methyl alcohol for 10 minutes, followed by blocking for 1 hour with normal swine serum in 0.3% Triton X-100. Cell cultures were incubated overnight at room temperature in the primary antibody, rabbit anti-TH (1:750; Pel Freeze, Rogers, AR). The cultures 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/H\(_2\)O\(_2\), 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 \(\times\) 0.25 mm divided into a 10 \(\times\) 10 grid) to count 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 coordinates (\(x,y\)) within the total culture area. All data were subjected to statistical analysis with two-way analysis of variance and two-sample t-tests.

Results

All groups yielded cultures that appeared healthy when examined using phase-contrast microscopy during the 48-hour culture period (Fig. 1). The percentage of TH-immunoreactive cells was increased in the GDNF-supplemented group for all hibernation periods when compared with the groups stored in hibernation medium without GDNF, but the increase was only statistically significantly higher in the 6- and 9-day groups (Fig. 2). This increase in the percentage of TH-immunoreactive cells was also significantly higher than in fresh controls. The percentage of TH-immunoreactive cells declined after 9 days in hibernation, becoming significantly less than that in fresh control cultures in the 15-day hibernation group.

Discussion

It is estimated that only 1 to 20% of grafted dopaminergic cells survive neural transplantation. As a result, a large number of cells—derived from multiple donor fetuses—is required for a transplant to produce meaningful functional outcomes.\textsuperscript{22,28,58} Because of the difficulties surrounding the acquisition of fetal donors and the limited supply of donors of the correct gestational age, storage of tissue is desirable to allow collection over several days. Furthermore, complete microbiological screening for infectious diseases in donor tissues is more easily accomplished when storage times are extended. Unfortunately, however, when cells are stored using methods such as frozen and cool storage for longer than 3 days, the result has been decreased graft volumes and decreased functional recovery in animal studies\textsuperscript{1,11,47,48} and clinical trials.\textsuperscript{41,50} This is thought to be due to increased cell fragility and susceptibility to oxidative stress.\textsuperscript{4,39}

Fortunately, there is evidence that TH-positive cell survival may be augmented by the addition of neuroprotective factors to the hibernation medium.\textsuperscript{1,15,30} Grasbon-Frodl, et al.,\textsuperscript{1} have demonstrated increased TH-positive cell survival and functional recovery in the rat model of PD by adding a lazaroid to the hibernation medium for stored cells. Further-
Fig. 1. Photomicrographs of rat fetal VM tissue in culture. Cells were cultured for 48 hours and then processed for TH immunoreactivity and double-stained with cresyl violet. Specimens include fresh control cells (A) and cells subjected to 6-day (B and C), 9-day (D and E), 12-day (F and G), and 15-day (H and I) hibernation with GDNF-supplemented medium (C, E, G, and I), and control medium (B, D, F, and H). Scale bar = 100 μm.
more, our group has previously demonstrated that adding GDNF to the hibernation medium as well as to the cell suspension before transplantation increases the absolute number of surviving TH-immunoreactive cells per graft, compared with using tissue that was untreated with GDNF or treated with GDNF only at the cell suspension stage. We subsequently demonstrated that adding GDNF to the hibernation medium resulted in increased fiber density in the zone surrounding the graft, and that it also resulted in 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 stored with GDNF.

In this study, we have examined GDNF-supplemented hibernation for extended time periods of 6 to 15 days. We found that GDNF-supplemented hibernation promotes a significant increase in the percentage of TH-immunoreactive cells when tissue is stored for up to 9 days at 4°C (p < 0.02), compared with both fresh control and non–GDNF-supplemented hibernation groups (this increase is not observed at 12 and 15 days [p > 0.05]). Our results indicate that storing fetal tissue in hibernation medium 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 that survive and will 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 patients receiving transplants.

Although the precise molecular mechanism(s) underlying the “dopaminotrophic” effect of GDNF remains unclear, a suggested mechanism is the suppression of caspase activity. Caspases are mediators of neuronal apoptosis, and reactive oxidative stress has been demonstrated to cause apoptosis through caspase activation. Hibernation of tissue may lead to the buildup of intracellular free radicals caused by ischemia, and reactive oxidative metabolites may be released during cell lysis. Supplementation with GDNF may thus prevent oxidative stress-induced apoptosis by preventing caspase activation.

It is likely that the antiapoptotic effect of GDNF involves protein synthesis through induction of nuclear transcription. Sawada and colleagues reported that simultaneous administration of GDNF with apoptosis-inducing neurotoxins to midbrain neurons failed to rescue neurons from the action of the toxins. This observation is in line with data from our group showing that GDNF-supplemented hibernation of VM tissue before transplantation led to significantly increased fiber outgrowth and the percentage of TH-immunoreactive cells per graft, when compared with transplants of fresh VM tissue grafted with (but not stored in) GDNF. These data seem to indicate 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-immunoreactive cell populations of VM stored for 24 hours, as determined using cell smear immunohistochemical studies, there was no difference in TH-immunoreactive cell populations in grafts of fresh tissue compared with tissue subjected to hibernation for 2 days. These authors suggested that there exists a population of TH-positive cells destined to die within the first 24 hours of hibernation or after grafting. Preincubation with GDNF in the cell hibernation medium may rescue this particular cell population from early cell death.

Conclusions

Glia 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 preventing the decline in human fetal VM TH-positive cell survival that is typically seen beyond 3 days in cool storage. Our study demonstrates that exposing rat VMs to GDNF during tissue hibernation increases the percentage of TH-immunoreactive cell populations for up to 9 days after extraction from fetuses, compared with fresh control cultures. This finding has practical implications for clinical neural transplantation because GDNF-supplemented hibernation of fetal VM cells will enable longer storage periods of graft material, which is a critical issue in neural transplantation for PD.

References

4. Collier TJ, Gallagher MJ, Sladek CD: Cryopreservation and stor-
age of embryonic rat mesencephalic dopaminergic neurons for one year: comparison to fresh tissue in culture and neural grafts. Brain Res 623:249–256, 1993


