Cryopreservation of Human Brain Tissue

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INTRODUCTION

Restoration of complex neurologic functions by transplantation of brain cells into rodents and primates (3, 4, 8) has generated interest in similar approaches for treating neurologic diseases in man. Human disease states in which specific neurochemical deficits exist, such as the neostriatal dopamine deficiency in Parkinsonism, seem to be an appropriate starting point at which to attempt to apply such methodology. Reversal of the clinical manifestations of Parkinsonism by medical therapy which increases central dopaminergic tone has been clearly documented (21). Evidence from dopamine-deficient rodent and primate models suggests that transplanted fetal dopamine-producing cells can also rectify the neurologic impairments (5, 6, 12). Improvements have also been reported after transplantation of different brain regions into animal models of Alzheimer's disease (3), Huntington's disease (8), and cortical blindness (17).

The possibility of transplantation of human fetal neural tissue is a logical extension of similar work in animal models (14, 19). Apart from the complex scientific and ethical (20) issues raised by such an approach, many practical problems need to be addressed regarding the safety, identity, and viability of the tissue, and the means by which the tissue could be preserved while safety testing was proceeding.

We have begun to resolve several of these questions using, as a prototype, ventral mesencephalic tissue collected from human fetal cadavers soon after death. Since brain tissue from rats (7, 9) and monkeys (1) retains viability and function during cryopreservation we assessed the utility of this method for preserving and holding human fetal cells. While the tissue was cryopreserved screening tests for the presence of infectious agents were carried out on adjacent brain tissue from the same fetus. We found that after thawing, cryopreserved mesencephalic cells retain high viability, express tyrosine hydroxylase, and synthesize dopamine.

METHODS

Tissue collection. Prior to scheduled abortion of normal first trimester (7–12 weeks old) fetuses, permission was obtained from the gravida to study cadaver tissues and cells according to a protocol approved by the Human Investigation Committee of Yale University School of Medicine. The ethical guidelines for this protocol have been previously described (14). Following vaginal preparation with Betadine sponges, dilation of the cervix was performed under local anesthesia. A number 10–12 cannula was used to evacuate the uterus. The sterile aspiration bottles containing the products of conception were brought to a tissue culture laboratory where they were emptied onto an autoclaved stainless steel screen and rinsed with iced, sterile 0.01 M phosphate-buffered saline (PBS). Midbrain fragments were identified by gross morphology and were then dissected with the aid of a dissecting microscope by one of us (C.L.) to obtain 1- to 2.5-mm³ pieces from the region of the substantia nigra (SN) and the ventral tegmental area (VTA). This area was identified by surface landmarks: the crus cerebri and the interpeduncular fossa (IF). Tissue from the middle third of the ventral mesencephalon rostral to the IF was collected. The tissue was held at 4°C in sterile test tubes containing PBS with 5 mM glucose and bubbled with carbogen to maintain a high oxygen concentration.

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Time from the end of the suction procedure to arrival of the tissue in the laboratory was 3-5 min; the collection and dissection of fragments required from 2 to 8 min. Time after collection of SN/VTA fragments on ice until freezing varied from 30 to 180 min. Viability of the cells, judged by trypan blue exclusion, did not vary within these time intervals.

Freezing and storage. Tissue blocks were prepared for freezing by exposing them to increasing concentrations of dimethyl sulfoxide (DMSO) in Hanks' balanced salt solution (HBSS) at 4°C in the dark. Ten-minute incubations in 0.25, 0.5, 1.0, and 1.5 M DMSO were performed. Blocks were then aspirated into freezing straws (IMV, Minneapolis, MN) and cooled to -6°C. Ice nucleation was begun away from the tissue, which was then held at -6°C for 30 min, after which the tissue was cooled in a controlled rate freezer (Planer Freezer Model R 204, TS Scientific) at a rate of 0.3°C/min until -80°C at which point the straws were rapidly immersed into liquid nitrogen. Some tissue blocks were frozen using propanediol as the cryopreservative according to the method of Testart and colleagues (18). Viability, as determined above, was always greater than 95% using this cryopreservation technique. When plated as described below, the propanediol-preserved cells grew as well as those preserved in DMSO.

Cell culture. Frozen tissue blocks in straws were rapidly thawed by immersion into a 37°C bath under sterile conditions. DMSO was cleared by repetitive rinses in culture medium (see below). The blocks were then incubated in a papain–neutral protease–DNase solution (10) in HBSS and dispersed by gentle trituration every 15 min for 45 min at 37°C. The cells were pelleted by low speed centrifugation, resuspended in culture medium (DMEM–Ham’s F-12 (1:1) with 20% fetal calf serum (FCS), and counted in a hemocytometer. In specimens which stayed completely submerged for 1 week to 3 months in liquid nitrogen, cellular viability was always greater than 90%. Viability was reduced (as low as 77%) in DMSO-cryopreserved tissues which had been repeatedly removed and replaced in the liquid nitrogen during the retrieval of other specimens. Cells were plated at 4–5 × 10⁶ cells/35 mm² dish in 2 ml of culture medium. Dishes or glass coverslips were coated with gelatin, poly-L-lysine, and FCS. The medium was changed every fourth day.

Immunohistochemistry. Seven-day-old SN/VTA cultures were examined for the presence of tyrosine hydroxylase-like immunoreactivity (THLI). Cells from the caudal brain stem (not including the SN/VTA) were cultured as controls. Cultures were rinsed in PBS at 4°C, fixed by immersion in 4% paraformaldehyde for 30 min at 25°C, and stained for THLI as previously described (2). The presence of THLI in freshly thawed SN/VTA blocks was examined. Three blocks, each from a different source, were thawed, immediately fixed with 4% paraformaldehyde, stained for THLI, as above, and flattened between a microscope slide and a coverslip ("squash-prep"). The presence of THLI was then determined by light microscopy.

Dopamine assay. Tissue dopamine content was determined as follows: Cells were collected and homogenized in extraction buffer (500 μl concd HClO₄ and 500 μl 10% sodium metabisulfite were diluted up to 50 ml with distilled water; dihydroxybenzylamine (DHBA) was added as an internal standard, to a final concentration of 0.5 pg/μl). After centrifugation at 3000 g for 5 min the supernatant was analyzed for dopamine and the pellet for DNA. A specially designed HPLC chromatographic system was used for analysis of dopamine. Narrow-bore (2.1 mm i.d.) columns were packed with 3 μm C 18 particles (Phase Separations, Norwalk, CT). The mobile phase was 0.2 M phosphate buffer containing octanesulfonic acid (200 mg/liter), 15% methanol, and 0.1 mM EDTA, pH 2.9. A battery-powered potentiostat was used for applying a potential to the glassy carbon detector electrode (Bioanalytical Systems, West Lafayette, IN). This HPLC system, along with more recent refinements (Bradberry and Roth, in preparation) allowed for routine limit of detection of 2–3 fmol; this level of sensitivity was necessary for the detection of dopamine in these samples. An alumina extraction procedure was performed prior to the HPLC analysis (15).

Microbiological testing. When SN/VTA tissue was identified by microdissection, adjacent tissue was transported on ice to the microbiology laboratories where it was homogenized in a tissue grinder and inoculated onto plates containing brain–heart infusion broth, blood agar, chocolate agar, thioglycollate broth, anaerobic blood agar, MacConkey’s medium, Mycotrim plates, or Sabauraud’s medium. Another adjacent tissue fragment was sent to the Yale Virology Laboratory where it was homogenized and plated in human placenta and foreskin lines, a human cervical carcinoma line (HEP2), a green monkey kidney line (VERO), and a rhesus kidney cell line to screen for a wide variety of viruses, including cytomegalovirus. In addition, in cases in which SN/VTA tissue was recovered, serum from the gravidae was screened for Hepatitis B and for HIV.

RESULTS

Products of conception from 120 pregnancies were examined. An intact midbrain, which allowed dissection of SN/VTA dopamine neurons with a high degree of confidence, was present in 32 cases. We found that an intact midbrain was most likely to be present and to be large enough for dissection of the SN/VTA in fetuses between 9 and 12 weeks of age. Following suction evacuation the midbrain of older fetuses was too disrupted for identifi-
cation of necessary landmarks. After thawing, tissue blocks of approximately 1 mm$^3$ yielded between 2.5 and 6 × 10$^5$ cells. For comparison an unfrozen 1-mm$^3$ SN fragment yielded 5 × 10$^5$ cells.

When fragments which had been frozen for 10–14 days were thawed, dispersed, and cultured, cells began to attach to the dishes by 2 h. Neurite extension was seen regularly by 5 h after plating. By 24 h, cells with the morphology of neurons were easily distinguishable from glial elements by phase-contrast microscopy. The neurons (Fig. 1) were phase-bright with perikaryal halos. Both fusiform and stellate phenotypes were present with highly branched processes (300–500 µm in length). Initially cultures were highly enriched in neuronal phenotypes; however, by 7 days in vitro (DIV) many glial elements had appeared and formed a basal monolayer upon which many large neuronal cells could be seen.

THLI-positive neurons were present in SN/VTA cultures after 7 days in vitro. Approximately 1% of all of the neuronal cells in vitro stained for THLI (see Fig. 2). These cells had many small dendrites and, in some cases, axons which extended more than five times the diameter of the perikaryon. In most instances, THLI was present throughout the entire length of the processes. By comparison, more than 50% of the immature cells in the “squash-preps” of freshly thawed 9- to 10-week-old SN/VTA blocks (n = 3) stained for THLI.

The presence of THLI suggested that the cells might be functional. To directly confirm that dopamine was being produced, three SN/VTA fragments were separately cultured (5 × 10$^5$ cells/well; n = 5 wells). At 10 DIV, cells from each fragment were separately harvested for measurement of dopamine. Cultures contained 117.9 ± 37 (mean ± SEM) pg dopamine/10$^6$ cells. The concentration of dopamine in the cells was 18.1 ± 4.2 pg/µg DNA. Cultures (n = 4) of brain fragments adjacent to the SN/VTA region were grown in a similar fashion; no dopamine was detected in these cells. For comparison, a 1-mm$^3$ SN/VTA fragment from a 9-week-old fetus was homogenized prior to cryopreservation and found to contain 9.3 pg of dopamine.

In the 32 cases examined, three tissues exhibited single organism bacterial growth of normal vaginal flora (coagulase-negative Staphylococcus, Bacillus species [not typed], and β Streptococcus group B). When the SN/VTA tissues from two of these cases were thawed and cultured, rapid bacterial overgrowth was found in both, confirming the utility of the bacteriologic screen-

![Phase-contrast photomicrograph at 200× of a 7 DIV culture of human ventral mesencephalic cells in monolayer culture. Both neuronal (thin arrows) and glial (arrowheads) elements are visible.](image)
FIG. 2. Photomicrograph of human fetal neurons which were stained for tyrosine hydroxylase-like immunoreactivity (THLI). Very long axonal and dendritic processes are visible in THLI-positive cells after 7 days in vitro.
ing procedure. All viral studies, including HIV, were negative. One patient had positive antibodies against hepatitis B; however, B surface antigen testing was negative.

**DISCUSSION**

These studies demonstrate that specific brain regions can be aseptically recovered from first trimester human fetal cadavers after suction abortion and, after cryopreservation, remain viable and retain the capacity to extend neurites and to produce region-specific neurochemical markers.

The ventral mesencephalon was chosen for our initial studies because it is a discrete area which can be identified by topographical landmarks. It also has characteristic developmental markers such as TH and dopamine. The SN/VTA is known to be involved in motor control in man, and damage to cells in this region in humans is associated with parkinsonism. Transplantation of this region into the brains of animals with induced dopamine deficiency corrects many of the neurologic defects. We have recently reported that human fetal SN/VTA tissue obtained and cryopreserved using these methods survives and develops when transplanted into monkeys (14).

These studies were undertaken with the rationale that if such tissue were to be utilized for human transplantation it would have to undergo safety testing of several weeks duration. Careful evaluation of potential candidates and surgical preparation of a recipient for the transplant would be optimized if the tissue could be made available to the surgeon at a precise time in a state of high viability. The fact that several tissue fragments (9%) were contaminated with normal vaginal bacteria indicates that microbiologic screening should be an important element of any program intending to use human fetal tissue for transplantation. The value of more stringent vaginal cleansing protocols in preventing such contamination must be established. Although we considered it unlikely that apparently normal fetal brain tissue would contain viral particles, we did screen for a wide range of vaginal and CNS viruses; in no case was a positive culture seen. The viral cultures employed cannot detect the presence of slow viruses. Since our viral cultures are normally held for 6 weeks, this is the minimum waiting period necessary for its potential use as a transplant.

We chose cryopreservation over cell culture as a holding method for several reasons. Cryopreservation preserves the normal cell-cell interactions present in the developing SN/VTA, which would be disrupted during dispersal of the tissue for cell culture. Second, biological processes such as aging proceed at undetectable rates at −196°C (11), whereas maturation and differentiation proceed in vitro, potentially limiting the ability to adapt to a transplant environment. Finally, unlike cells in culture, cryopreserved blocks do not become infected while in liquid nitrogen nor do they have to be disrupted again prior to use in transplantation.

The use of either DMSO or propanediol resulted in high levels of viability of the thawed tissue. The immunohistochemical studies indicate that dopaminergic neurons survive cryopreservation while retaining the ability to extend dendrites and axons and to express a neuronal phenotype. The immaturity of this area at 9–12 weeks of age may explain the low tissue concentrations of dopamine. The fact that only 1% of the neuronal cells, after 7 DIV, express THLI as compared to the 50% level in the “squash-preps” prior to culture may represent damage during the tissue dispersal, lack of appropriate neurotrophic factors in the tissue culture medium, or transient expression of TH in a larger number of fetal cells which is lost during differentiation. It is important to point out that the survival in vitro in no way predicts the survival in vivo, especially with regard to potential viability in parkinsonian striatum.

The presence of dopamine in the SN/VTA cultures and not in cultures of adjacent non-SN/VTA tissue confirms that the correct tissue was collected and that the THLI represents functional TH. The presence of picomolar concentrations of dopamine in the cells after 10 DIV demonstrates the ability of these cells to mature rapidly in a foreign environment after cryopreservation. Studies are underway to examine the ability of tissues held long term (13 months to 2 years) to grow in vitro and to produce THLI and dopamine.

We believe that the ability to retrieve precisely, and characterize carefully, human neural tissues will markedly improve the chances for success of cellular replacement therapy in neurodegenerative diseases. However, the potential of human fetal neurons to reverse the neurologic manifestations of a human neurodegenerative disease such as parkinsonism remains speculative. Although fetal SN/VTA tissue can produce dopamine and survive in animals with damage to substantia nigra (13), there are certainly other cellular and humoral elements in these tissue blocks which contribute important factors for the development of dopamine neurons and which may provide signals necessary for normal nigrostriatal function. The demonstration that transplanted fetal monkey SN can ameliorate the MPTP-induced parkinsonism in monkeys (16) sets the stage for considering such an approach in humans with parkinsonism. However, the etiologic process responsible for the destruction of dopamine cells in parkinsonism may still be active and may destroy freshly implanted cells. Moreover, if the normal postsynaptic targets of dopamine have been destroyed then even viable dopamine-producing cells may not have any effect.

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