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Medical Terminations of Pregnancy: A Viable Source of Tissue for Cell Replacement Therapy for Neurodegenerative Disorders

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"Proof-of-principle" that cell replacement therapy works for neurodegeneration has been reported, but only using donor cells collected from fetal brain tissue obtained from surgical terminations of pregnancy. Surgical terminations of pregnancy represent an increasingly limited supply of donor cells due to the tendency towards performing medical termination in much of Europe. This imposes a severe constraint on further experimental and clinical cell transplantation research. Therefore, we explore here the feasibility of using medical termination tissue as a donor source. Products of conception were retrieved from surgical terminations over the last 7 years and from medical terminations over the last 2.5 years. The number of collections that yielded fetal tissue, viable brain tissue, and identifiable brain regions (ganglionic eminence, ventral mesencephalon, and neocortex) were recorded. We studied cell viability, cell physiological properties, and differentiation potential both in vitro and following transplantation into the central nervous system of rodent models of neurodegenerative disease. Within equivalent periods, we were able to collect substantially greater numbers of fetal remains from medical than from surgical terminations of pregnancy, and the medical terminations yielded a much higher proportion of identifiable and dissectible brain tissue. Furthermore, we demonstrate that harvested cells retain the capacity to differentiate into neurons with characteristics appropriate to the region from which they are dissected. We show that, contrary to widespread assumption, medical termination of pregnancy-derived fetal brain cells represent a feasible and more readily available source of human fetal tissue for experimental cell transplantation with the potential for use in future clinical trials in human neurodegenerative disease.

Key words: Human fetal tissue; Neural transplantation

INTRODUCTION

Cell replacement therapy involves the transplantation of human fetal neuroblasts, obtained from elective terminations of pregnancy, into the brains of patients with neurodegenerative diseases such as Parkinson's (PD) and Huntington's disease (HD) (17,27). Following transplantation, implanted neuroblasts can mature, innervate the surrounding neuropil, and contribute to the repair of circuitry that has been damaged as a result of the degeneration. Several clinical trials for both PD and HD have demonstrated proof-of-principle that cell replacement therapy can provide significant alleviation of symptoms in both conditions (1,19,22).

Empirically, the most successful transplants are taken from the developing fetal brain close to the time of birth of the specific populations of neurons to be transplanted (18). At this stage the fate of the neurons is determined, but they retain the developmental plasticity required to integrate into the adult host environment. As a consequence, tissue for transplantation must be harvested within a specific gestational time window, and from the precise region in the developing (fetal) brain that contains the cells lost in the disease process. For example, the dopamine containing neurons of the ventral mesencephalon (VM), and the medium spiny neurons (MSNs) developing within the fetal ganglionic eminence (GE—putative striatum), for PD and HD, respectively, are born

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in the developing human brain towards the end of the first trimester [VM between 4 and 6 weeks (30) and GE between 6 and 9 weeks postconception (pc), respectively (26)].

To date, successful clinical trials have utilized fetal tissue from surgical terminations of pregnancy (STOP). We have been collecting tissue from STOP for cell research and replacement therapies since 1997 using low-pressure transvaginal aspiration, with research ethical committee approvals and with informed maternal consent (26). However, STOP tissue of suitable quality is in limited supply due to several factors. Routine high-pressure aspiration of fetal material does not yield identifiable brain tissue, so that successful collection requires engagement of an interested surgeon willing to gain experience with low-pressure aspiration for collection of tissues for research use. Moreover, even low-pressure aspiration results in substantial tissue fragmentation, variable tissue quality, and difficulty in standardization of collection and dissection procedures. The pressure on supply has been compounded by the requirement for multiple fetal donors per patient in order to achieve satisfactory cell numbers for some applications such as PD neural transplantation (18), and more recently by the move throughout Europe towards replacing STOP with medical termination of pregnancy (MTOP) (5,7). MTOP is induced by administration of prostaglandin and antiprogesterone drugs over a 48-h period, and the fetus is usually passed during the following 8–12 h. In our own center, this has resulted in STOP tissue becoming an extremely scarce resource over the last 2–3 years.

Collection from spontaneous pregnancy loss has already been shown to be unsuitable for use in human transplantation (3,11), and the use of MTOP material was not previously pursued due to the assumption that the pharmacological agents used to induce termination and the unspecified period of fetal death prior to expulsion would adversely affect the viability of the collected tissues. However, on the basis of experimental analyses, these assumptions can be challenged, and here we demonstrate good viability of MTOP-derived fetal brain tissue in experimental cell transplantation. These new data pave the way for detailed preclinical functional screening to assess the suitability of MTOP-derived fetal cells for clinical neural transplantation studies.

MATERIALS AND METHODS

Human fetal tissue was collected following the guidelines of the Polkinghorne (23) and Department of Health (6) reports and with Bro Taf Local Research Ethics Committee approval. Full consent was obtained from the maternal donor, following consent for the termination, as part of the MRC-sponsored, South Wales initiative for transplantation (SWIFT) program. Fetal tissue ranged in age from 6 to 12 weeks pc, assessed by ultrasound measurements and confirmed using fetal morphometric data upon termination (8,14).

Surgical Termination of Pregnancy

Tissue was collected via low-pressure aspiration using an Ipas manual vacuum aspirator and cannulae (Durbin Clinic Sales, Middlesex, UK), with the aid of transvaginal ultrasound. Following extraction, the products of conception were discharged into 50-ml centrifuge tubes containing phosphate-buffered saline (PBS) supplemented with glucose (0.6%) and heparin (5 μg/ml). Collected material was transferred to a petri dish for inspection, and fetal parts transferred to a labeled 50-ml centrifuge tube containing commercial hibernation medium (Hibernate E, Invitrogen, Strathclyde, UK), and transported on ice to the laboratory.

Medical Termination of Pregnancy

Mifepristone (200 mg) was administered orally and the patient admitted to the gynecology ward 36–48 h later, at which time 800 μg misoprostol was self-administered transvaginally. Following expulsion of the products of conception into a disposable cardboard receptacle, fetal material was inspected by the nursing staff, was promptly transferred to a sterile 500-ml pot containing Hibernate E, and was stored at 4°C (usually for up to 3 h but for a maximum of 12 h) before being transported on ice. The time at which the products of conception were passed were recorded.

Assessment of Gestational Age

The age of the fetus was estimated using three methods: i) ultrasound in the clinic, ii) crown–rump length (CRL) of the tissue in the laboratory, and iii) when the fetal remains were fragmented (more common in STOP products), CRL was estimated using a formula based on measurement of fetal limbs and other body parts (8,12).

Dissection and Preparation of Human CNS Tissue

Tissues of interest (VM, GE, and cortex) were dissected and tissue pieces were stored overnight in Hibernate E at 4°C for processing the following day. The small number of embryos that were collected on the day following termination were stored as whole embryos in Hibernate E overnight at 4°C and dissected and processed the following morning.

Preparation of Cell Suspension

Dissected tissue pieces were incubated in 200 μl of either TrypLE express (Invitrogen, Paisley, UK) or 0.1% bovine trypsin (Worthington, UK) for 20 min at 37°C, after which either benzonase (Merck, UK, 1:10,000) or DNAase (Sigma, UK, 0.01%) was added, and tissue in-

cubated for a further 5 min at 37°C. VM tissue was then placed in 200 µl of fresh DNAase for trituration. GE and cortical tissue were washed twice with Dulbecco's modified Eagle's medium (DMEM/F-12, supplemented with 1% PSF antibiotic-antimycotic), and then collected by centrifugation at $100 \times g$ for 3 min. The tissue was resuspended in 200 µl DMEM/F12, and then triturated to produce a single cell suspension.

Assessment of Cell Viability

Cell suspensions from each region of interest were counted in a hemocytometer using trypan blue exclusion. The proportions of live and dead cells were recorded and viability expressed as the percentage of live cells in the suspension (21). Statistical analysis was carried out with GenStat statistical software. Mean comparison was performed using a *t*-test.

To assess differentiation potential, dissociated cells were plated onto 13-mm, poly-L-lysine-coated coverslips (0.1 mg/ml in distilled H_2O) at a density of 1×10^5 cells/well. Cells were placed in the center of the coverslip in 30 µl of differentiation medium (standard medium plus 1% fetal calf serum and 2% B27) and allowed to differentiate for either 3 days (VM cells) or 7 days (GE cells), and fed by replacing half the medium with fresh medium every 3 days. Cells were then washed using PBS and prepared for immunocytochemistry by fixation with 4% paraformaldehyde in 0.1 M PBS for 20 min. For calcium imaging the cells were maintained in differentiation media for up to 24 days.

Indirect fluorescent immunocytochemistry was performed using standard protocols (15) with primary antibodies directed against β-III tubulin (1:1000, Sigma), glial fibrillary acidic protein (GFAP, 1:1000, DAKO), dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32, 1:1000, Chemicon) and the dopamine marker tyrosine hydroxylase (TH, 1:1000, Chemicon), and appropriate fluorescent-labeled secondary antibodies. Fluorescent staining was visualized on a Leica DRMB microscope, and cell counts performed at 400× magnification. For eachcoverslip, at least five random fields were counted. Pseudocolor fluorescent images were obtained using Openlab 2.1 image analysis software.

Experimental Animals

Animal experiments were performed in full compliance with local ethical guidelines and approved animal care according to the UK Animals (Scientific Procedures) Act 1986. Sprague-Dawley (Harlan UK) rats were used as graft recipients, housed four per cage in a 12 h:12 h light/dark cycle, and with access to food and water ad libitum.

Immunosuppression of animals receiving xenografts of human fetal cells was achieved either by neonatal desensitization (for the GE grafts) (16), or by daily injections of cyclosporine (10 mg/kg, IP) commencing the day before transplantation until the day of sacrifice (VM grafted animals), depending on the availability of prepared host animals at the time of tissue collection. For neonatal inoculation host animals were inoculated IP with human fetal CNS cells on the day of birth. Upon reaching adulthood (220–250 g), rats received unilateral quinolinic acid lesions of the neostriatum (standard model of HD; $n = 6$), prior to receiving intracerebral transplants of GE cells by stereotaxic injection into the neostriatum at 10 weeks of age. Rats receiving VM grafts ($n = 4$) were "hemiparkinsonian," having been unilaterally lesioned using the neurotoxin 6-OHDA, to induce >95% dopamine depletion on one side of the brain. VM graft tissues were implanted into the dopamine-depleted striatum on the lesioned side of the brain.

Transplantation

Dissociated suspensions of VM cells were resuspended in 0.01% DNAase in DMEM/F-12 at 300,000 cells/2 µl. GE cells were resuspended at 500,000 cells/2 µl.

Rats were anesthetised with gaseous 1.5–3% isoflurane in a 2:1 O_2/NO mixture, mounted in a Kopf model 900 stereotaxic frame. All animals received 2 µl of cell suspension injected via a 10-µl glass microsyringe targeted at the right dorsal striatum, coordinates: anterior in front of bregma, AP = +0.6 mm; lateral to the midline, L = −2.6 mm; vertical below dura, V = −5.5 and −4.5 mm (1 µl at each depth); with the nose bar set −2.3 mm below the interaural line. Graft tissue was injected over 2 min, with a further 3 min allowed for diffusion before the needle was slowly withdrawn, the wound cleaned and sutured, and the animal allowed to recover. All animals received a 5-ml SC injection of 0.18% sodium chloride with 4% glucose (Aqupharm, Dunnington, York) to avoid dehydration. Paracetamol analgesia was administered via the water supply for 2 days following operation.

Posttransplant Immunohistochemistry

Animals were sacrificed either 4 weeks (VM transplants) or 12 weeks posttransplantation; all animals were sacrificed by barbiturate overdose (Euthatal, Merial Animal Health, Harlow, UK) and perfused via the ascending aorta with 50–100 ml 0.1 M PBS (pH 7.5) followed by 250–300 ml 4% paraformaldehyde in PBS. Brains were removed, cryoprotected in 20% sucrose, and sectioned at 40 µm on a freezing sledge microtome. A 1:6 series of free-floating sections was stained immunohistochemically using the biotin-streptavidin-horseradish peroxidase method. Primary antibodies against the following antigens were used: human nuclear antigen (HuNu, 1:500, Chemicon), DARPP-32, (1:1,000, Chemicon), and

TH (1:1000, Chemicon). Stained sections were inspected and photographed using a Leica DRMB microscope with an Optronics Magnafire digital camera, under bright field illumination.

Quantification of Graft Parameters

Graft volume and cell number calculations were based on HuNu staining and determined using the Olympus CASTgrid stereology system on a 1:12 series. The fractionator method was used that samples a fraction of the total cells (i.e., the fraction of the area samples, the fraction of sections stained, and the ratio between the section thickness and the dissector from which they were counted) (29). Graft volume was calculated for each experimental group.

Calcium Imaging

Cells were loaded with 6 μM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, USA) diluted in normal DMEM/F-12, for 30 min at 37°C. Cells were placed in a perfusion chamber mounted upon an Olympus IX71inverted microscope equipped with a Cairn monochromator-based fluorescence system (Cairn Instruments, Faversham, Kent, UK) and were continuously superfused with a solution containing in (mM) 135 NaCl, 5 KCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES, 10 glucose, pH 7.4. Fura-2 was alternately excited at 340 and 380 nm. Images at 510 nm were acquired at 0.2 Hz by a slow-scan CCD camera (Kinetic Imaging Ltd, Nottingham, Notts UK). Following background subtraction, emission ratios (340/380) were calculated off-line using the Andor IQ 1.3 software package (Andor Technology, Belfast, UK). γ-Aminobutyric acid (GABA) (50 μM), 50 μM kainate, 50 μM α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), 50 μM *N*-methyl-D-aspartic acid (NMDA), and 100 μM L-glutamic acid (all Sigma-Aldrich, Poole, UK) were rapidly (20 ms solution exchange) applied locally to the cells using gravity-driven perfusion through a multibarrel motorized solution exchanger (Bio Logic Rapid Solution Changer RSC-100; Molecular Kinetics, Pullman, WA, USA). A depolarizing solution (in mM) 90 NaCl, 50 KCl, 1.25 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES, 10 glucose (pH 7.4) was used to determine neuronal excitability.

RESULTS

The SWIFT program commenced in October 2003 and by the end of the period of study in March 2009, 300 patients undergoing STOP consented to donate embryos to the program. However, since 2007, in Cardiff as in the rest of the UK, the number of early terminations by STOP has declined markedly. Both the numbers and the proportion of early terminations by MTOP have correspondingly increased. Consequently, in the last 2 years (January 2007 to March 2009) we have recruited 300 cases from the MTOP list. The following analysis is based on a comparison of the first 300 cases collected by each method, a milestone reached at the same time for both procedures in March 2009.

Tissue Collection

Table 1 highlights the numbers of cases that were analyzed and identifies the differences between STOP and MTOP tissue collections in relation to the number of useable tissues harvested from the two sources. Of the 600 cases consented, 17.6% STOP and 22% MTOP withdrew, either from the termination itself (in most cases) or from consent to donation. There were 13.7% MTOP donations lost as a result of the hospital protocol in which patients under 7 weeks pc were allowed home to complete the procedure if expulsion had not taken place on the ward during the course of the day. A small percentage of samples were lost at collection (0.8% and 10% STOP and MTOP, respectively) for a variety of less common reasons, including: nonexpulsion of the fetus requiring emergency surgery; failure in labeling and quality control; and transportation difficulties. There were 9.3% STOP and 29.6% MTOP samples estimated on collection to be outside the inclusion age range, which was less than 10 weeks pc, and so were not taken for subsequent analyses.

Of the samples collected, 43% STOP and 87.5% MTOP contained identifiable fetal parts from which measurements could be taken. The age of the fetus was estimated independently, based on the Evtouchenko equation (8), which was found to correlate closely with the age as estimated from ultrasound measurement in vivo ($r = 0.910$, $df = 126$, $p < 0.001$). This is directly comparable to the correlation, $r = 0.875$, previously reported for STOP (26).

A substantially greater proportion of MTOPs yielded identifiable CNS tissue than did STOPs (94% MTOP compared to 39.6% STOP). Furthermore, in most MTOP, the CNS tissue included brain, whereas a large proportion of the STOP yielded spinal cord with no identifiable brain tissue. This is likely to be related to the greater fragmentation seen in STOP. Specifically, STOP samples yielded fewer than 5% (5/134) intact fetuses, with most samples being significantly fragmented. By contrast, the fetus was entirely intact and in some cases still within the amniotic sac in more than 90% (114/120) of MTOP. As a consequence, we were able accurately to dissect cortex, VM, and GE from the majority of cases (see Table 1). Figure 1 presents the same data, expressed as a percentage of total MTOP or STOP consented in order to illustrate the ultimate yield of spe-

Table 1. Differences Between STOP and MTOP Tissue Collections in Relation to the Number of Useable Tissues Harvested From the Two Sources

	STOP	MTOP
Total number consented	300	300
Subsequently withdrew	53/300 (17.6%)	66/300 (22%)
Sent home to complete	n/a	32/234 (13.7%)
Not collected or missed by ward*	2/247 (0.8%)	20/202 (10%)
Too large for purpose	23/245 (9.3%)	54/182 (29.6%)
Measurable fetal parts	96/222 (43%)	112/128 (87.5%)
No brain tissue	134/222 (60%)	8/128 (6.2%)
CNS	88/222 (39.6%)	120/128 (94%)
Ctx	64/88 (73%)	114/120 (95%)
GE	40/88 (45%)	112/120 (93%)
VM	22/88 (25%)	103/120 (86%)

A total of 600 patients have consented to donate tissue for use in the SWIFT program. Over time the actual number of tissue samples is much lower and the table highlights where samples were lost. Where samples were over the 10 week pc age at the time of passing the tissue was deemed too large for our purposes and so was not included for analyses. The last three rows in the table show the percentage of tissue samples from which regions of interest were obtained and are expressed as a percentage of all collected samples as opposed to a percentage of all that consented. STOP, surgical termination of pregnancy; MTOP, medical termination of pregnancy; Ctx, cortex; VM, ventral mesencephalon; GE, ganglionic eminence.

*Missed samples include those not collected by the team, patients who failed the MTOP requiring evacuation of retained products, or those who were not identified as South Wales initiative for transplantation (SWIFT) on the ward and subsequently not collected.

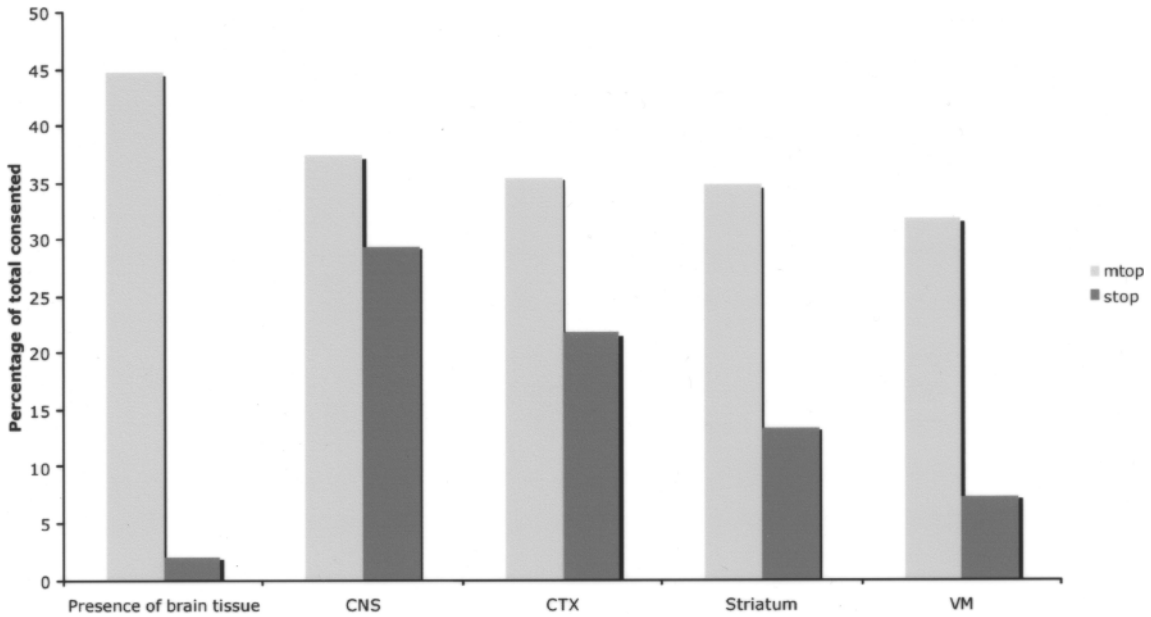

Figure 1. The number of cases where identifiable tissue was retrievable from both medical (MTOP; light gray) and surgical terminations of pregnancy (STOP; dark gray). The dissection accuracy from MTOP tissue was far greater than that from STOP tissue due to the fragmented nature of STOP tissue.

cific brain regions of interest from the two TOP methods, indicating the much higher yields derived from MTOP compared to STOP.

Cell Viability

The utility of the tissue, whether for research or therapy, depends on not only supply but also the viability of the tissue obtained. Cell viability was assessed in all dissociated CNS tissues by trypan blue exclusion. Overall, the viability of MTOP neurons was somewhat lower than that derived for STOP [means ± SEM, 75.73 ± 1.24% vs. 85.28 ± 0.89%, respectively, $t(174) = 5.399$, $p < 0.0001$] (Fig. 2). Previous experience has indicated that an initial trypan blue exclusion higher than 80% [(22) and our unpublished results] results in good cell viability both in vitro and in vivo. Forty-nine percent of MTOP samples and 85% of STOP samples exceeded this criterion, but the lower proportion of viable samples from MTOP was markedly offset by the much higher rate of retrieval of brain areas of interest (e.g., 58/103 VM from MTOP vs. only 9/22 VM from STOP, from a total of 300 initial consents in each case).

Characterization of MTOP Tissue

In addition to trypan blue evaluation of viability of the freshly prepared cells, we evaluated the long-term viability of MTOP tissue for survival and differentiation by three additional criteria (Figs. 3–5). First, MTOP-derived tissue was seen to differentiate into appropriate populations of neurons when cultured in vitro. In particular, cultures derived from VM expressed TH, a marker of surviving dopamine neurons (Fig. 3A) and tissue from the GE-expressed DARPP-32 as a marker of MSNs (Fig. 3B). The percentage of total cells that are DARPP-32 positive in cultures is in the range of 60–75%, which represents 75–90% of all neurons from primary WGE tissue in the range of 20–30 mm CRL. This is comparable to what we have reported previously from STOP-derived GE tissues. In the case of VM tissue the number of TH-positive neurons from each VM was calculated according to the age of the tissue piece. It was found that the number of TH neurons ranged from 50,000 to 200,000 cells per VM, which is in accordance to previously reported studies using STOP-derived tissue. Secondly, we have used calcium imaging as a measure of neuronal differentiation and health, and find comparable profiles of functional cellular activity from the two tissue sources (Figs. 4 and 5). Specifically, at 24 days in vitro (DIV) cells derived from both MTOP (Fig. 4A) and STOP (Fig. 4B) responded to the nonselective depolarizing stimulus of 50 mM extracellular KCl (high K^+ 1 with a rise in intracellular calcium concentration ($[Ca^{2+}]_i$). The mean rises in $[Ca^{2+}]_i$ in the cell populations derived from MTOP and STOP were almost identical at around 30% above baseline (Fig. 4C). The mean increases in $[Ca^{2+}]_i$ that were evoked in the two populations by NMDA, GABA, AMPA, kainite, and glutamate were, again, almost indistinguishable (Fig. 4C), with NMDA evoking the most robust rises in both cases (Fig. 4A, B, C). Furthermore, after 15 min of recording, and following five further applications of agonists, the responses to a subsequent depolarizing stimulus (high K^+ 2) evoked increases in $[Ca^{2+}]_i$, which were not significantly diminished from those evoked by the

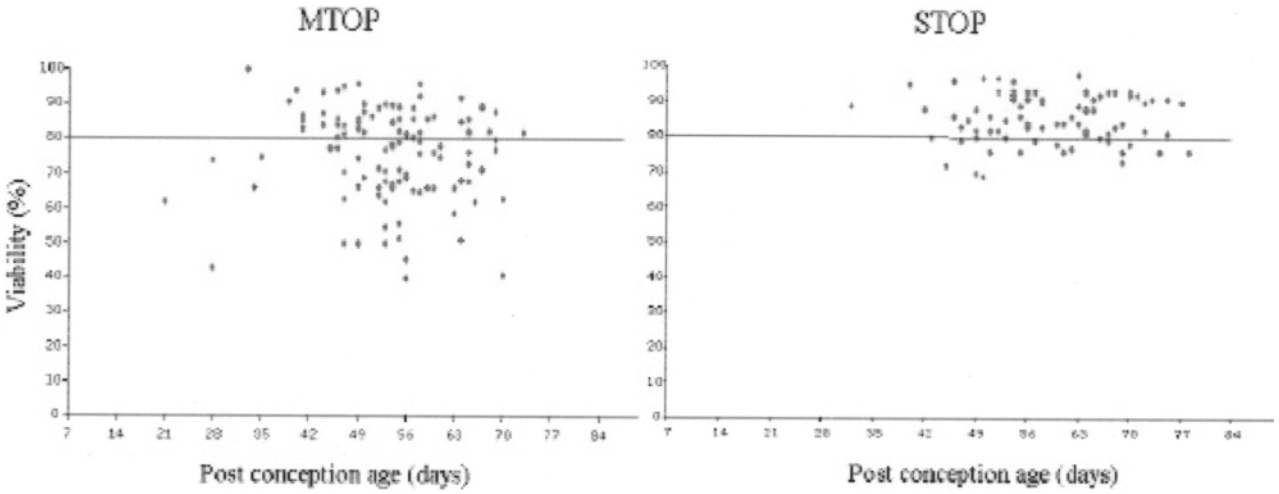

Figure 2. The viability of the tissue (one data point per fetus) was assessed using a trypan blue exclusion assay. The trend line shows the proportion of tissue with viability of 80% and above; this is the minimum viability currently accepted for use in clinical transplantation paradigms. For MTOP tissue 58% were above the 80% cut-off whereas for STOP tissue the percentage is much higher at 82%.

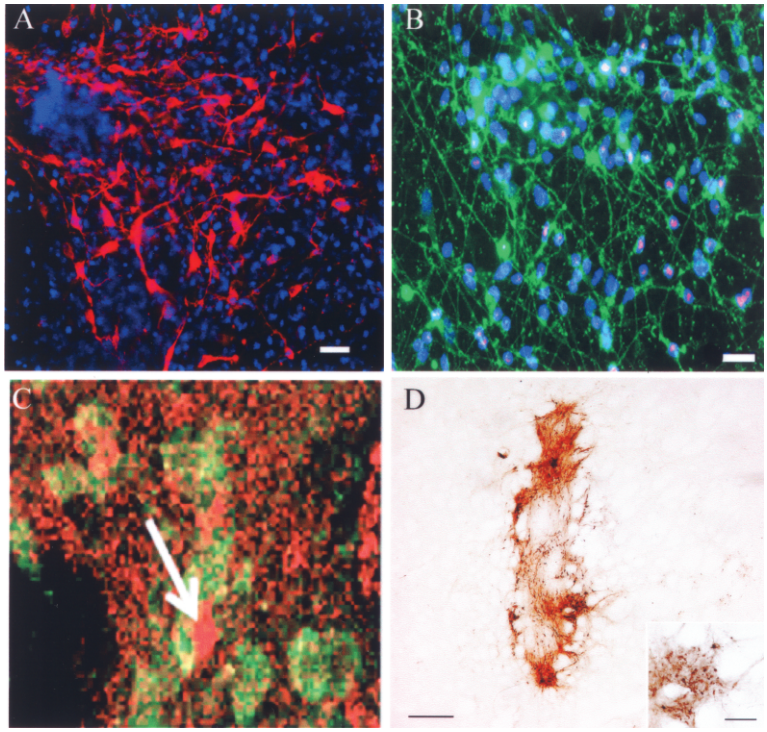

Figure 3. Photomicrographs of immunohistochemical labeling for (A) tyrosine hydroxylase (TH; red) in MTOP-derived 3-day in vitro differentiated ventral mesencephalon (VM) cells, and (B) dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32; green) 7-day in vitro differentiated MTOP-derived ganglionic eminence (GE) cells, highlighting the potential of MTOP-derived tissue to differentiate in tissue culture into neurons specific to its region of origin. Blue represents Hoechst staining. Immunohistochemical staining of (C) DARPP-32 (green)/human nuclear antigen (HuNu; red)-positive neurons within the graft 12 weeks posttransplantation. At this survival time DARPP-32-positive neurons are seen principally at the periphery of the graft. (D) TH-positive neurons within the graft 4 weeks posttransplantation. Inset: Higher power of TH-positive neurons. Scale bar: 50 μm.

first depolarizing stimulus (Figs. 4C, 5). This indicates that the smaller responses seen upon application of agonists were not a result of any type of experimentally induced, time-dependent dysregulation in calcium homeostasis per se. The cell-by-cell analyses shown in Figure 5, which individually plot the responses evoked by the first depolarizing stimulus against the responses evoked by each individual agonist (and second depolarizing stimulus) of each cell, indicate that MTOP and STOP essentially respond similarly to this range of agonists. Thus, the regression lines of the increases in $[Ca^{2+}]_i$ evoked by high K^+ 1 versus high K^+ 2, GABA, AMPA, kainite, and L-glutamate are not significantly different from each other.

Thirdly, both VM and GE cell suspensions survived transplantation into the relevant animal lesion models, yielding large healthy grafts in all animals, with DARPP-32-positive (Fig. 3C) or TH-positive neurons (Fig. 3D), respectively, directly comparable to previous reports of human fetal xenografts derived from STOP (15). The mean graft volume was 0.52 mm^3 in the case of GE-grafted tissue (STOP graft volume mean = 0.69 mm^3) and 0.68 mm^3 in VM tissue grafts. However, the number of mature cells within the graft was somewhat different for the two tissue sources with a mean of 16 cells/mm^3 DARPP-32-positive cells in the GE grafts compared to 7173cells/mm^3 for VM grafts. The low number of mature DARPP-32-positive cells in the GE grafts is most likely due to short survival time, as it has been shown previously that such grafts require long time points in vivo for full maturation to take place.

DISCUSSION

The shift from STOP to MTOP in recent years has imposed severe limitations on STOP as a source of tissue for both experimental and clinical studies of cell replacement therapy in CNS neurodegeneration. Contrary to presupposition, we have here identified MTOP tissue as a viable source of human fetal cells for transplantation studies in neurodegenerative disorders such as PD

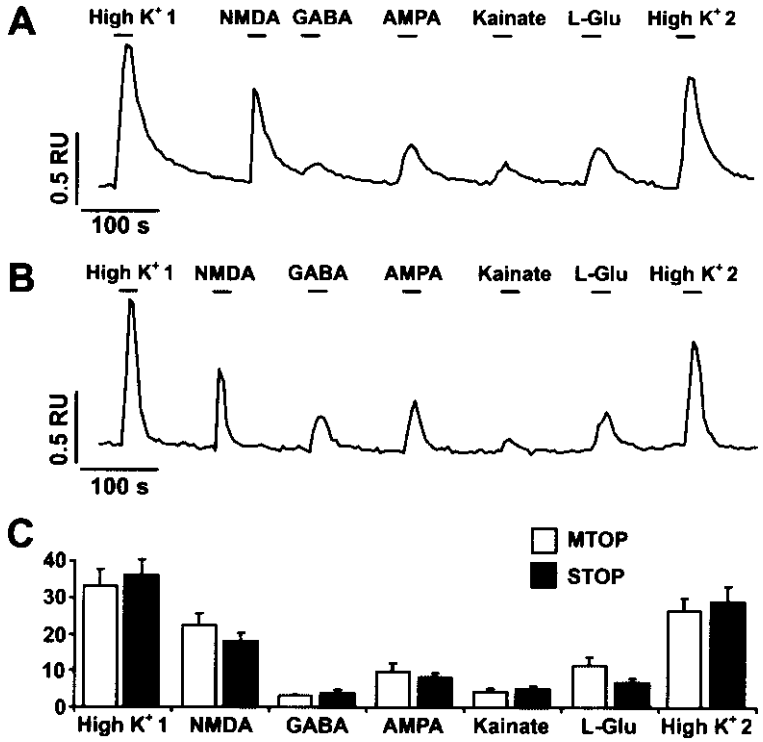

Figure 4. Intracellular calcium concentration $[Ca^{2+}]_i$ rises evoked by depolarizing stimuli and neurotransmitter applications. Rises in intracellular calcium in exemplar, individual cells derived from MTOP (A) and STOP (B) fetal GE tissue during brief applications of depolarizing solutions (high K^+ 1 and high K^+ 2), 50 μM γ-aminobutyric acid (GABA), 50 μM kainate, 50 μM α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), 50 μM *N*-methyl-D-aspartic acid (NMDA), and 100 μM L-glutamic acid. Relative $[Ca^{2+}]_i$ is reported as the ratio of Fura-2 emission intensities at 510 nm following excitation of the probe sequentially at 340 nm and 380 nm and is plotted as relative units (R.U.) versus time. Agonists were applied for the durations indicated by the bars above each trace. (C) Mean ± SEM rises in $[Ca^{2+}]_i$ in response to each agonist and high K^+ solutions plotted as percentage increase in fluorescence above baseline [$n = 230$ MTOP (open bars), $n = 88$ STOP (filled bars).

and HD. The process of a MTOP is undertaken over a 48-h period, raising the possibility that the fetus could die at any time during that period. Thus, the concern that the CNS cells could be subject to a prolonged period of anoxia, rendering them nonviable for transplantation purposes, is fully plausible. Although a proportion of MTOP collections do indeed show lower viability than STOP cells, the vast majority were over 50% viable, and 58% were in the acceptable range (≥80% viability). In addition, it may be that a broader viability range proves to be acceptable, and experimental studies are ongoing to assess this question. Importantly, the greater variance in viability was more than off-set by the substantially greater number of MTOP cases that could be collected over a given period of time in addition to the increased number of intact brain retrievals from the MTOP cases (allowing easy identification of particular brain regions). This is of importance because not only is the accuracy of dissection an imperative for a successful transplant, but implantation of unwanted tissues can have detrimental consequences (10,20).

A crucial requirement of transplanted cells is that they are able to differentiate into the precise phenotype lost to the disease process. We show here for both VM and GE tissues that this can be achieved in vitro. The number of region specific neurons (TH and DARPP-32) generated from respective brain regions was comparable to that previously reported for STOP-derived tissue (4,14).

Where both STOP and MTOP GE tissues were directly compared by calcium imaging, it was found that the majority of the neurons responded to all of the agonists used, a good indicator that they are functional neurons. NMDA, AMPA, kainite, and L-glutamate are all linked with striatal neuron function. The remarkable similarities in the responsiveness of MTOP and STOP neurons suggest that neuroblasts derived from both sources survived the procurement process equally well and were able to develop into mature and functionally

active neurons. Of particular note are the responses to GABA. It is well documented that fetal, in contrast to adult, central neurons respond to GABA by depolarizing. This so-called fetal excitatory response of $GABA_A$ receptor activation is due to the fact that the equilibrium potential of chloride (the permeant ion of $GABA_A$ receptor channels) of fetal neurons is more positive than the fetal resting membrane potential (2). That both MTOP and STOP cells respond to GABA by increasing $[Ca^{2+}]_i$ demonstrates that GABA evokes a depolarization of a magnitude sufficient to cause voltage-activated calcium influx, and indicates that both MTOP- and STOP-derived cells in vitro still express a fetal phenotype, even after 24 DIV. The responses to NMDA are interesting in that MTOP cells demonstrate a slightly larger population than STOP cells, which are clearly excitable (in that they respond to high K^+ 1) but do not respond robustly to NMDA (Fig. 5, upper central panel). Currently, we have no explanation for this slight difference, but because the regression lines (MTOP vs. STOP) are identical if these particular cells are excluded from the analysis, it appears that there is a small subpopulation of cells (ca. 10%) from MTOP that are not present in STOP cells; whether this subpopulation is selectively ablated in the STOP preparation or whether it is an artifact of the MTOP procedure is currently a matter of conjecture. However, work is ongoing to characterize further these responses over time in vitro, which should give insight into such questions in the near future.

Following transplantation into the appropriate animal models, we also see evidence of differentiation appropriate to the area from which the donor tissue was derived. Due to the scarcity of useable STOP tissue in recent times, these grafts have been compared to grafts of STOP-derived striatal tissue performed in this laboratory using identical conditions (26) or previously published data (4,13,14,28). Although a direct comparison would have been more satisfactory, the paucity of available STOP tissue is likely to be a continuing situation, and indeed is the rationale for this study in the first place. The low numbers of DARPP-32-positive cells are generally what is found at this survival time point and it is likely that longer periods posttransplantation are needed to allow full maturation of GE grafts.

We have shown here that MTOP-derived tissue is a viable source of cells for experimental transplantation studies in HD and PD. The use of such tissue overcomes the logistical issues associated with the present and future use of STOP-derived tissue. The greater spread in the viability of the MTOP-derived tissue is overcome by the greater supply of this tissue. Previously, we have reported on infectious data collected from STOP tissue (9) and similar data collection is now ongoing for MTOP tissue with a view to this tissue being used for

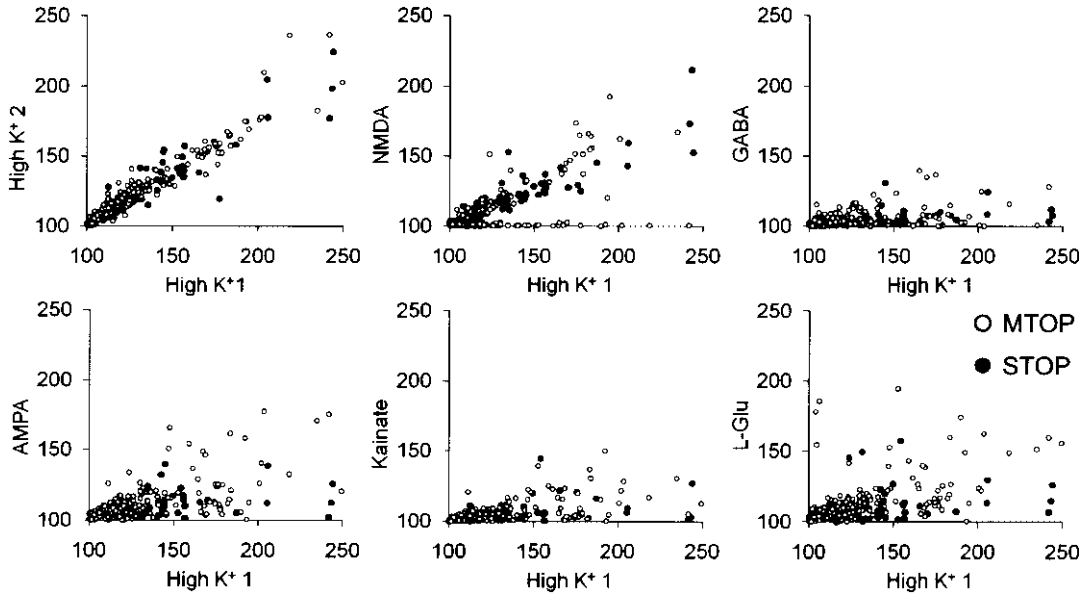

Figure 5. Cell-by-cell analysis of intracellular calcium concentration $[Ca^{2+}]_i$ rises evoked by each individual neurotransmitter. Rises in $[Ca^{2+}]_i$ evoked by the second depolarizing stimulus (high K^+ 2) and each agonist (50 μM GABA, 50 μM kainate, 50 μM AMPA, 50 μM NMDA, and 100 μM L-glutamic acid) are plotted against the rises in $[Ca^{2+}]_i$ evoked in the same cell by the first depolarizing stimulus (high K^+ 1). As in Figure 4C, relative $[Ca^{2+}]_i$ was assayed as the ratio of Fura-2 emission intensities at 510 nm following excitation of the probe sequentially at 340 and 380 nm and is plotted as percentage increase in fluorescence above baseline [n = 230 MTOP (open symbols), n = 88 STOP (filled symbols)].

human therapeutics. The infectious risk from STOP tissue is very low (9) and theoretically that from MTOP tissue may be lower, as the fetus is frequently retrieved within the amniotic sac.

The data presented here suggest that MTOP tissue may be as suitable as STOP tissue for future clinical application in neurodegenerative disease, and indeed may ultimately prove to be superior in that it can be more readily and reliably dissected. The suitability for clinical application will, of course, require further studies to demonstrate functional benefit of MTOP-grafted tissue in disease models. Functional benefit has been demonstrated previously for human STOP-derived VM tissue (24,25), but it is interesting to note that there have been little functional data to date for GE-grafted animals, in part due to fall in STOP tissue availability over recent times.

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