NEUROGASTROENEROLOGY

Characterisation and transplantation of enteric nervous system progenitor cells

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Two supplemental figures are available at <http://gut.bmj.com/supplemental>.

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Revised 4 August 2006
Accepted 11 August 2006
Published Online First
14 September 2006

Gut 2007;56:489–496. doi: 10.1136/gut.2006.094565

Aims: Enteric nervous system (ENS) progenitor cells have been postulated to be an appropriate source of cells for the treatment of Hirschsprung's disease. In order for this to be successful, the techniques previously used for the isolation of rodent ENS progenitor cells need to be adapted for postnatal human tissue. In this paper, we describe a method suitable for the preparation of both mouse and human postnatal ENS progenitor cells and assess their transplantation potential.

Method: Single cell suspensions were isolated from 11.5 days post-coitum embryonic mouse caecum and postnatal human myenteric plexus. These cells were cultured under non-adherent conditions to generate neurospheres which were implanted into aganglionic embryonic mouse hindgut explants. Cell proliferation, migration and differentiation were observed using immunofluorescence microscopy.

Results: Neurospheres generated from both mouse and human tissues contained proliferating neural crest-derived cells that could be expanded in tissue culture to generate both glial cells and neurons. When implanted into aganglionic murine gut, cells migrated from the neurospheres using pathways appropriate for cells derived from the neural crest, and differentiated to become glia and neurons expressing neuronal phenotypic markers characteristic of the ENS including nitric oxide synthase and vasoactive intestinal polypeptide.

Conclusion: We have developed a technique for the isolation and expansion of ENS progenitor cells from human neonates. These cells have the ability to differentiate into neurons and glia when transplanted into aganglionic gut, this demonstration being a necessary first step for their autologous transplantation in the treatment of Hirschsprung's disease.

The enteric nervous system (ENS) not only controls the peristaltic activity of the gut but also regulates many other functions including secretion, mucosal blood flow and immunity.¹ Most neurons and glia of the ENS originate from vagal neural crest stem cells that enter the proximal gut during early embryonic life and migrate distally to colonise the entire bowel.² A sacral contribution of neural crest cells colonises the bowel in the opposite direction to the level of the vitello-intestinal duct, although these cells are relatively few in number and are limited principally to the hindgut.³ Incomplete gut colonisation by the neural crest cells results in Hirschsprung's disease, characterised by aganglionosis of the distal bowel.⁴ It has been shown that multipotent, self-renewing ENS progenitor cells (ENSPC) capable of generating neurons and glia derived from the neural crest can be isolated from the gut of fetal and neonatal mice and rats, and are capable of colonising the colon after transplantation.^{5–8} These findings raise the prospect of using such ENSPC to treat children with Hirschsprung's disease.

Neural stem cell isolation has been previously achieved by immunoselection based on expression of cell-specific markers^{5,6,9,10} and also by the generation of neurospheres.^{8,11} Neurospheres comprise aggregates of stem cells and their progeny that arise when cells dissociated from neural tissue are cultured and proliferate to form cell clusters which subsequently develop as aggregates in suspension under non-adherent conditions.¹² When applied to cells dissociated from fetal and postnatal rodent gut, this technique has been used to isolate ENSPC which differentiate to produce neuronal and glial progeny when transplanted into cultured gut explants.⁸ Recently, preliminary evidence has been presented that neurosphere-like bodies may be generated from cells dissociated from human gut tissue, although the differentiation and

transplantation of cells from the neurosphere-like bodies remains to be established.¹³

In this paper we describe the development of a modified technique suitable for the generation of neurospheres from both murine and neonatal human ENSPC. We show that the neurospheres generated from these cells contain multipotent cells by demonstrating their proliferative potential and differentiation of neuronal and glial progeny. We further demonstrate that these cells can be transplanted into aganglionic embryonic bowel where they migrate and differentiate into both neurons and glia. The ability of neonatal human ENSPC to be grown in culture and subsequently transplanted is a prerequisite for the development of autologous stem cell therapies for Hirschsprung's disease.

METHODS

Generation of mouse neurospheres

Time mated CD-1 mice (Charles River Laboratories UK, Kent, UK) were sacrificed by cervical dislocation in accordance with UK Home Office regulations. Caeca from 11.5 days post-coitum (dpc) embryos were dissected under aseptic conditions into sterile Dulbecco's modified Eagle medium (1 mg/ml glucose) (DMEM, Gibco, Invitrogen, Paisley, UK), in which they were maintained at 37°C until dissociation.

Abbreviations: BrdU, bromodeoxyuridine; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; dpc, days post-coitum; DMEM, Dulbecco's modified Eagle medium; ENS, enteric nervous system; ENSPC, enteric nervous system progenitor cell; FITC, fluorescein isothiocyanate; GDNF, glial cell line-derived neurotrophic factor; HRNP, human ribonucleoprotein; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PGP9.5, protein gene product 9.5; SP, substance P; TH, tyrosine hydroxylase; VIP, vasoactive intestinal polypeptide

Figure 1 Generation of neurospheres from mouse ENSPC. Freshly dissociated 11.5 days post-coitum caecal cells shown by (A) phase contrast and (B) immunofluorescence for p75. Aggregates of cells after (C) 5 days and (D) 30 days in culture. (E–H) Sections of a 30 day neurosphere showing immunofluorescent labelling of (E) BrdU incorporation, (F) p75, (G) PGP9.5 and (H) S100B. Calibration bars: (A–C) 50 μ m, (D–H) 250 μ m.

Caeca were dissociated in 0.025% (w/v) trypsin-EDTA (Gibco) dissolved in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS, Gibco) for 45 min at 37°C. To halt digestion, the trypsin solution was replaced with 2 ml culture medium. The caeca were triturated using a fire-polished glass Pasteur pipette, to give a suspension of single cells that were counted using a haemocytometer. The culture medium was DMEM (1 mg/ml glucose) containing 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco), supplemented with 2 mM glutamine (Gibco), 7.5% (v/v) chick embryo extract (Sera Laboratories International, Horsted Keynes, UK), 20 ng/ml epidermal growth factor (EGF, Sigma-Aldrich, Poole, UK), 20 ng/ml FGF2 (Sigma-Aldrich), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich), 1% (v/v) N1 medium supplement (Sigma-Aldrich) and 1% (v/v) fetal calf serum (Gibco). Cells were cultured in 35 mm bacteriological Petri dishes (Sarstedt, Leicester, UK). At 3 day intervals, mildly adherent cells were gently resuspended by agitation and the culture medium containing non-adherent cells was transferred to a new dish and replenished with an equal volume of fresh medium.

Figure 2 (A–D) Proliferation and differentiation of single mouse neurosphere cells. An individual cell dissociated from a 21 day old primary neurosphere photographed sequentially after (A) 12 h, (B) 7 days and (C,D) 10 days in culture. (D) Merged image of immunofluorescence photomicrographs of the colony double-labelled for PGP9.5 (red) and S100B (green). Note the localisation of S100B-positive cells along the neurites (arrows) as well as within the aggregate. (E–H) Primary neurosphere cultured on a fibronectin substrate. Immunofluorescence photomicrographs show (E) NOS after 7 days, (F) VIP after 7 days, (G) SP after 7 days and (H) CGRP after 10 days. Calibration bars: (A, B) 25 μ m, (C–E) 50 μ m, (F–H) 20 μ m.

To expand the neurosphere population, the primary neurospheres derived directly from caecal cells were collected after 21 days of culture, dissociated and cultured as for the caecal cells. The secondary neurospheres so generated were counted and this process was repeated to generate tertiary neurospheres.

Generation of human neurospheres

Approval for the use of human tissue was obtained from the Liverpool Research Ethics Committee. Human ENSPC were isolated from three patients with diagnoses of Hirschsprung's disease (from ganglionic bowel), imperforate anus and colonic atresia, with an age range of 3 weeks to 7 months. In each case, a 1 cm² sample of bowel was removed, wrapped in sterile, saline-soaked gauze and placed in a sterile container at room temperature. The sample was transported to the laboratory and processed within 30 min. To obtain human neurospheres, the mucosa and submucosa were dissected free from the circular

and longitudinal smooth muscle layers. The muscle layers were cut into small pieces (1–2 mm²) and incubated in a solution of 0.5% (w/v) collagenase/0.5% (w/v) dispase (Gibco) supplemented with 2.5 mM Ca²⁺ for 1 h at 37°C. The tissue was then gently triturated and centrifuged at 800 rpm for 5 min. The supernatant was removed and replaced with fresh collagenase/dispase solution at 1 h intervals and the process repeated 2–4 times until the tissue was almost fully reduced to a single cell suspension. The culture medium used for human cells was identical to that used for the generation of mouse neurospheres, but supplemented with 100 µg/ml metronidazole (Sigma-Aldrich).

The resulting cell suspension was filtered through a 40 µm filter (Falcon Cell Strainer, BD Biosciences, Oxford, UK) and cultured using the same protocol as for mouse neurospheres.

Differentiation of neurospheres

Eight-well slides (BD Falcon CultureSlides, BD Biosciences) were sequentially coated with 150 µg/ml poly-D-lysine (Sigma-Aldrich) for 8 h and 50 µg/ml bovine fibronectin (Sigma-Aldrich) for 2 h. Single neurospheres were collected using a mouth pipette, placed into individual wells and cultured for up to 10 days in 200 µl culture medium. The culture medium was topped up every 2 days but not changed to avoid dislodging small colonies. Where indicated, glial cell line-derived neurotrophic factor (GDNF, Sigma-Aldrich) was added to the medium at a final concentration of 10 ng/ml.

Neurosphere volume was calculated using the formula $v = \frac{4}{3}\pi r_1^2 r_2$, where r_1 was the smallest radius of the cell when viewed using phase contrast microscopy and r_2 was the larger radius (assuming an ellipsoid shape; if the neurosphere was perfectly round then $r_1 = r_2$). These radii for all neurospheres present in 35 mm Petri dishes were measured 0, 7, 14, 21 and 28 days after the start of culture. A two-tailed *t* test was used to determine the significance of any differences between groups at each time point.

Transplantation of neurospheres into aganglionic gut

Specimens of distal hindgut were harvested from 11.5 dpc CD-1 mouse embryos and cultured on a semi-permeable membrane (Millicell, Millipore, Watford, UK) as previously described.¹⁴ Single mouse or human neurospheres that had been cultured for a minimum of 21 days were apposed to the proximal end of the isolated hindgut. As controls, neurospheres were placed next to explants of 11.5 dpc mouse liver, and gut explants were cultured without neurospheres. Specimens were fixed and processed for immunohistochemistry after 4 and 8 days in culture.

Immunohistochemistry

All specimens were fixed in 4% (w/v) paraformaldehyde in PBS for 1 h at room temperature and then rinsed extensively with PBS. Neurospheres and gut explants were immersed in 20% (w/v) sucrose for 1 h and overnight, respectively, at 4°C, prior to embedding in 7.5% (w/v) gelatine (Sigma-Aldrich) in 15% (w/v) sucrose. Embedded specimens were frozen at –80°C in isopentane (Sigma-Aldrich) and serial sections cut using a cryostat (7 µm neurospheres, 10 µm bowel).

For immunostaining, samples were pre-treated for 30 min with PBS containing 1% (w/v) bovine serum albumin (Sigma-Aldrich) and 0.5% (w/v) Triton X100 (VWR, Poole, UK) except prior to p75 labelling when the Triton X100 was omitted. Primary antisera were applied for 16 h at the following concentrations: rabbit anti-p75 nerve growth factor receptor (p75, Abcam, Cambridge, UK) 1:400, rabbit anti-protein gene product 9.5 (PGP9.5, Biogenesis, Poole, UK) 1:4000, rabbit anti-S100B (Dako, Ely, UK) 1:200, rabbit anti-nitric oxide synthase

(NOS, Biogenesis) 1:40, sheep anti-tyrosine hydroxylase (TH, Abcam) 1:1000, rabbit anti-vasoactive intestinal polypeptide (VIP, Chemicon, Harrow, UK) 1:800, rabbit anti-calcitonin gene-related peptide (CGRP, Abcam) 1:50, rabbit anti-substance P (SP, Chemicon) 1:500, sheep anti-choline acetyltransferase (ChAT, Abcam) 1:400 and mouse anti-human ribonucleoprotein antibody (HRNP, Chemicon) 1:50. For PGP9.5/S100B dual labelling, guinea pig anti-PGP 1:400 (Chemicon) was used. Slides were rinsed twice with PBS before incubating for 4 h with appropriate secondary antisera as follows: fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Sigma-Aldrich), Cy3-conjugated donkey anti-guinea pig IgG (Chemicon) and FITC-conjugated rabbit anti-sheep IgG (Dako). After rinsing with PBS, the samples were mounted using Dako mounting medium.

To assess cell proliferation, 30 day old primary neurospheres and colonies of cells differentiating from adherent neurospheres after 5 days in adherent culture conditions were incubated with 10 µM bromodeoxyuridine (BrdU, BD Biosciences). After 4 h incubation, cells were fixed as described above, placed in 4 M HCl for 15 min and then washed with distilled water followed by PBS.¹⁵ Human neurospheres were incubated with 10 µM BrdU for 18 h before transplantation. Incorporated BrdU was detected by a 2 h incubation with mouse anti-BrdU (Dako) diluted 1:25 followed by rinsing in PBS and a further 2 h incubation with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich) diluted 1:200.

RESULTS

Preparation and characterisation of mouse neurospheres

The single cell suspension generated from dissociated 11.5 dpc mouse caecum contained a small population (<5%) of neural crest-derived cells identified by expression of p75¹⁶ (fig 1A,B). The majority of non-p75 expressing mesenchymal cells adhered to the bacteriological dishes a few hours after initial plating (not shown). By 5 days under non-adherent culture conditions, some of the suspended cells had proliferated to form neurosphere-like aggregates (fig 1C) that continued to increase in size up to at least 30 days (fig 1D; also see supplemental fig 1 available at <http://gut.bmj.com/supplemental>).

BrdU incorporation confirmed the presence of proliferating cells mainly restricted towards the neurosphere periphery (fig 1E). In contrast to the low numbers of p75-positive cells in the original caecal dissociate, the majority of neurosphere cells expressed p75, indicating their neural crest origin (fig 1F). Expression of the ubiquitous neuronal marker PGP9.5 demonstrated the presence of a dense neuronal network within the spheres (fig 1G). Small numbers of S100B-positive glial cells were scattered throughout the neurospheres (fig 1H).

When these primary neurospheres were dissociated and their component cells re-cultured, a further generation of secondary neurospheres with similar structural and differentiative properties developed. This process was repeated to generate tertiary neurospheres. With each neurosphere dissociation and subsequent re-culture, the number of proliferating neurospheres increased exponentially (table 1).

Multipotentiality of mouse neurosphere cells

To determine whether neurospheres contain multipotential progenitor cells, primary neurospheres grown in suspension culture for 21 days were collected and dissociated to generate single cell suspensions. Single cells were then cultured in individual wells of microtitre plates (fig 2A). A small number (<1%) of cells began to proliferate, forming aggregates after 7 days in culture (fig 2B). By 10 days, some of these neurosphere-like aggregates became loosely attached to the wells and

Figure 3 Migration and differentiation of mouse neurosphere cells into embryonic gut explants. 21 day primary neurospheres were apposed to 11.5 dpc hindgut and cultured for up to 8 days before processing. (A) p75 immunoreactivity during colonisation of the gut explant at 4 days. (B) p75 immunoreactivity during colonisation of the gut explant at 8 days. NS and L indicate the position of the neurosphere and explant lumen, respectively. (C) p75 immunoreactivity of control gut explant without transplanted neurosphere after 8 days. (D) Neurosphere apposed to liver explant, p75 immunofluorescence after 8 days. (E) Neurosphere applied to aganglionic hindgut explant after 8 days in culture stained for PGP9.5. Note the numbers of PGP positive cells within the bowel wall. (F) S100B immunoreactivity. (G) NOS immunoreactivity. (H) TH immunoreactivity. (I) VIP immunoreactivity. (J) CGRP immunoreactivity. Calibration bars: (A–F) 200 μ m, (G, J) 100 μ m, (H, I) 50 μ m.

fibres and cells began to migrate from the aggregates along the surface of the plate (fig 2C). Immunofluorescence for PGP9.5 confirmed that the fibres were neuronal processes and S100B immunofluorescence revealed small glial cells distributed along the length of these neurites (fig 2D). Thus, a small number of cells present within ENS neurospheres are proliferating multipotent neuronal and glial cell precursors. Furthermore,

Figure 4 The growth of human neurospheres. Phase contrast micrographs of (A) single cell suspension of human cells at D0, (B) neurospheres at D7 of culture and (C) neurosphere at D14 of culture. Note neurite outgrowth from the neurosphere (arrowhead). (D) Neurospheres at D28 of culture. (E, F) 21 day old human neurospheres after 10 days in culture on chamber slides treated with poly-D-lysine and laminin and stained for PGP9.5. (E) Neurosphere grown in standard culture medium without GDNF supplementation. (F) Neurosphere grown in standard culture medium with GDNF supplementation at 10 ng/ml. Calibration bars: (A, B, D) 50 μ m, (C, E, F) 100 μ m.

neurosphere-derived cells developed a variety of neuronal phenotypes: clusters of NOS-positive cell bodies were particularly dense and frequent (fig 2E). VIP- and SP-positive cells tended to occur in sheets, but occasional small groups were present (fig 2F,G). Infrequent single CGRP-labelled cells developed in the cultures after 10 days (fig 2H). No TH-positive cells were seen in differentiating colonies or the parent neurosphere at any time point.

Transplantation of mouse neurosphere-derived ENSPC into aganglionic hindgut

Four days after neurosphere transplantation, chains of p75 positive neural crest cells extended from the neurosphere within the gut wall for approximately 75% of the length of the hindgut (fig 3A). After 8 days, the cells had reached the caudal extent of explant, and were present at high density along its entire length (fig 3B). Control 11.5 dpc mouse hindgut explants did not contain any p75-positive neural crest cells after 8 days of culture, confirming the absence of endogenous enteric neural precursors (fig 3C). Furthermore, neurosphere-derived cells did not enter 11.5 dpc liver (fig 3D) or lung (not shown).

After 8 days in culture, neurosphere-grafted hindgut showed extensive PGP9.5 labelling (fig 3E). Only a small number of

Figure 5 Human neurospheres after 28 days in culture. (A) p75 immunoreactivity. (B) PGP9.5 immunoreactivity; inset shows detail of neuronal structures within the neurosphere. (C) S100 immunoreactivity. (D) BrdU immunoreactivity after 18 h incubation with 10 μ M BrdU immediately prior to collection; inset shows merged photomicrographs B and D showing cells within the neurosphere positive for PGP9.5 alone, BrdU alone and PGP9.5 and BrdU together (arrowhead). (E–I) Immunoreactivity for: (E) NOS, (F) ChAT, (G) VIP, (H) SubP, (I) CGRP. Calibration bars: 50 μ m.

S100B positive glial cells were present in the proximal hindgut after 4 days of culture, but after 8 days they had colonised the full length of the explant (fig 3F). After 4 days, NOS-positive cell bodies and fibres were present (fig 3G), and a very small number of cells displayed TH immunofluorescence (fig 3H). VIP- and CGRP-positive cells were not identified in day 4 post-transplant bowel but were present after 8 days (fig 3I,J). SP-positive neurons were not identified at either time point (not shown).

Preparation and characterisation of human neurospheres

As with mouse caecum, the single cell suspension generated from dissociated human colon contained a small population of neural crest-derived cells identified by expression of p75¹⁷ (not shown). When grown in non-adherent culture conditions, clusters of cells resembling neurospheres began to form and grow (fig 4A–D). The increase in volume of the human neurospheres did not significantly differ from that of the

mouse during culture (two-tailed *t* test, $p > 0.05$) (see supplemental fig 2 available at <http://gut.bmj.com/supplemental>).

When grown in standard culture medium, human neurospheres adhered to the culture dish if allowed to settle and would occasionally produce neurite-like processes (fig 4C, arrowhead). When cultured in chamber slides coated with extracellular matrix molecules to induce differentiation, neurite outgrowth was minimal (fig 4E), in contrast to neurospheres derived from embryonic mouse bowel (fig 2D,E). However, the addition of GDNF to the culture medium under these conditions stimulated marked neurite outgrowth from the human neurospheres (fig 4F).

Differentiation of neural cells

Immunohistochemistry confirmed the presence of the neural crest cell marker p75 (fig 5A) as well as glial and neuronal cell markers within mature neurospheres (fig 5B,C). BrdU incorporation demonstrated proliferating cells that tended to be uniformly distributed throughout the neurospheres, in contrast

Figure 6 Migration and differentiation of human neurospheres in mouse hindgut explants. The position of the grafted neurosphere is indicated by a solid arrowhead. (A) Phase contrast photomicrograph showing initial neurosphere position in relation to proximal hindgut on day 1 of culture. (B) Phase contrast photomicrograph showing position of human neurosphere (arrowhead) and growth of explant after 8 days in culture. (C–J) Immunoreactivity after 8 days in culture. (C) PGP9.5. The position of the transplanted neurosphere is indicated by the arrowhead and PGP9.5 positive cells can be seen distributed within the wall of the gut. L indicates the lumen of the explant. (D) Human nuclei on same specimen as shown in fig 5C. The inset shows a magnified view of the area indicated by the box, where a human cell nucleus that has migrated away from the original neurospheres graft can be seen. (E) BrdU immunoreactivity from the same explant as shown in fig 5C and D. Migration of cells with BrdU positive nuclei along the wall of the hindgut explant can be seen (hollow arrowhead). The inset shows a cell dual labelled for PGP9.5 (green) and BrdU (red). (F) Dual labelling showing PGP9.5 (green) and HRNP (red) positive cells that have migrated away from the neurosphere (top left) and along the wall of the bowel. Inset shows a detail of several cells positive for both PGP9.5 and HRNP. (G) Dual labelling for S100 (green) and HRNP (red) showing some, but not all, migrated cells expressing a glial phenotypic marker. (H) Immunoreactivity for ChAT. ChAT (green) and HRNP (red) dual labelling is shown in the inset. (I) Cells that have migrated away from the main neurosphere body dual labelled for NOS (green) and HRNP (red). Note the presence of NOS-positive, HRNP-negative cells within the bowel wall. (J) Migrated cells dual labelled for VIP (green) and HRNP (red). Calibration bars: (A–D) 200 μ m, (E) 100 μ m, (F–H) 50 μ m, (I, J) 20 μ m.

Table 1 Yield data for neurospheres generated from cells dissociated from embryonic murine caeca and from primary and secondary neurospheres

Cell source	Cell number per caecum (A) and per neurosphere (B, C)	Number of neurospheres isolated	Neurospheres generated per cell (%)	Cumulative yield of neurospheres
A. Caecum	90 595 ± 29 636	73 ± 24	0.08 ± 0.030	73
B. Primary neurosphere	10 750 ± 1061	30 ± 3	0.275 ± 0.007	2190
C. Secondary neurosphere	8500 ± 2121	30 ± 7	0.350 ± 0.141	65 700

Results are expressed as mean ± SD. The cumulative number of neurospheres isolated is stated per original caecum. The expansion of neurosphere numbers is approximately exponential.

to the embryonic mouse neurospheres (fig 5D compared to fig 1E). Dual labelling with PGP9.5 and BrdU showed the presence of cells positive for both BrdU and PGP, indicating that new neuronal cells had been generated from actively dividing precursors within the neurosphere (fig 5D inset, arrowhead).

Immunofluorescent labelling for neurotransmitters commonly found in the ENS showed the presence of NOS (fig 5E), ChAT (fig 5F), VIP (fig 5G), SP (fig 5H) and CGRP (fig 5I) within the neurospheres. No TH immunoreactivity was seen and the proportion of cells staining for NOS and ChAT was greater than that for the other neurotransmitters.

Transplantation of human neurosphere-derived stem cells into aganglionic mouse hindgut

Human neurospheres were transplanted onto the rostral end of cultured 11.5 dpc murine hindgut explants. Upon transplantation, fusion with the explant rapidly occurred and cells migrated into the mouse tissue (fig 6A,B). After 8 days in culture, staining for PGP9.5 showed the site of neurosphere engraftment and cells migrating within the bowel wall in a similar pattern to that seen with embryonic mouse neurospheres (fig 6C). To confirm that the migrating cells originated from the human neurospheres, they were dual-labelled with the marker of human cell nuclei, HRNP¹⁸ (fig 6D). In addition, neurosphere cells labelled with BrdU before transplantation could be seen to have migrated away from the neurosphere (fig 6E). Dual labelling with PGP9.5 and BrdU showed that cells which had undergone division within the neurosphere prior to transplantation were capable of migrating within the explant and differentiating into a neuronal phenotype (fig 6E, inset). Dual labelling with PGP9.5 or S100 and HRNP confirmed that cells within the neurosphere were capable of migration within the wall of the gut and differentiation into neuronal and glial phenotypes (fig 6F,G), indicating their origin as ENSPC born in the neurosphere.

ChAT-positive (fig 6H), NOS-positive (fig 6I) and a smaller number of VIP-positive cells (fig 6J) had migrated into the explants after 8 days in culture, but no TH-, CGRP- or SP-positive cells were detected (not shown). A consistent finding in these experiments was that NOS-positive but HRNP-negative staining cells were often found alongside NOS-positive, HRNP-positive cells (fig 6I).

DISCUSSION

We describe a method to obtain ENSPC from both embryonic murine and postnatal human intestinal tissue. Furthermore, we show that when cultured as neurospheres these cells proliferate and that they migrate and differentiate into neuronal and glial phenotypes on transplantation into aganglionic gut explants.

The human postnatal neurospheres were derived from gut tissue of patients with various diagnoses and ages; of particular interest was the ability of ENSPC obtained from the ganglionic bowel of a child with Hirschsprung's disease to generate neurospheres. This is consistent with the notions that the

ability of ENSPC from patients with Hirschsprung's disease to develop into neurons is not intrinsically defective, and that the neural progenitor cells remain throughout the bowel during early postnatal life, supporting the feasibility of autologous transplantation.

Recently, concerns have been raised about the use of BrdU staining to track stem cell engraftment.¹⁹ When injected into the brains of embryonic mice or adult rats, labelled BrdU stem cells or BrdU containing medium could give false positive results due to take up of BrdU from dead cells or directly from the medium. However, we demonstrate the presence of HRNP staining closely correlated with that of BrdU in engrafted specimens, indicating the migration of human ENSPC generated within neurospheres that subsequently differentiate to neuronal and glial phenotypes.

We observed that while the cultured human and mouse neurospheres contained cells with similar patterns of neurotransmitter expression, after transplantation fewer neuronal phenotypes appeared to have migrated within the gut tissue as no evidence of immunoreactivity for TH, CGRP or SP was found. This observation is consistent with the hypothesis that the differentiated neurons observed within the gut tissue originated from undifferentiated precursors in the human neurosphere, rather than being previously-differentiated neurosphere cells. Evidence has been presented consistent with a restriction in the developmental potential of postnatal rodent ENSPC.⁵ However, the presence of CGRP containing neurons within the untransplanted neurospheres supports the hypothesis that the progenitor cells are capable of producing cells from the Mash-1 independent lineage^{20, 21} and therefore have retained their pluripotency. Although there is a transient wave of TH expression during early ENS development,²² within the mature bowel intrinsic dopaminergic neurons (that also express TH) arise late in development from a Mash-1 independent precursor.²³ The lack of these neurotransmitters in cells migrating from our transplanted human neurospheres may therefore reflect the absence of appropriate signalling cues in the embryonic bowel.

The origin of the NOS-positive HRNP-negative neurons adjacent to the NOS-positive HRNP-positive cells that had migrated into the explants remains to be determined. Although it is known that not all human cell nuclei stain positive for HRNP,²⁴ the relatively large numbers of these NOS-positive cells may indicate that these cells originated from the sacral neural crest²⁵ but alone lack the capacity to form neurons in the absence of vagal ENSPC.

Clearly, demonstration that ENSPC can also colonise postnatal aganglionic bowel will be a necessary future step leading to their autologous transplantation in the treatment of Hirschsprung's disease. However, it should be noted that the postnatal bowel constitutes a markedly different environment from that found in the embryonic gut and knowledge of the molecular basis of Hirschsprung's disease, and the response of ENSPCs to growth factors such as GDNF, may be required for autologous transplantation to be successful. Furthermore,

before transplantation becomes a realistic possibility, experiments are necessary to determine the functionality of ENSPC-derived neurons. Interestingly, functional changes in the stomach of NOS deficient mice have recently been demonstrated using progenitor cells isolated from the subventricular zone of embryonic mouse forebrain.²⁶ Although this technique does not allow autologous grafting, the demonstration of similar functional changes using human-derived postnatal ENSPC will be an important step. Moreover, full characterisation of the behaviour of ENSPC and their progeny in the environment of the gut wall will be required in order to assess the safety of their transplantation in terms of absence of neoplastic or otherwise uncontrolled growth. Despite these reservations that can only be answered by future experimental work, the experiments described in this paper clearly show that not only is it possible to isolate and amplify human ENSPC, but that when transplanted into the embryonic gut wall these cells migrate and differentiate.

ACKNOWLEDGEMENTS

This work was supported by grants from the Birth Defects Foundation, Action Medical Research, The Children's Research Fund, and CORE (the Digestive Disorders Foundation), London, UK.

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Competing interests: None.

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From the question on page 455

An abdominal CT scan showed a target-like lesion in the ascending colon and dilatation of the small intestine consistent with intussusception. During surgery, an ileocolic intussusception complicated with gangrene was found. An ileal lipoma, corresponding to the yellow head observed during colonoscopy, was found to be the leading point. The patient had an uneventful recovery after surgery.

Intestinal intussusception in children is a common disorder; however, it is a rare clinical entity in adult patients. A demonstrable aetiology is found in 70–90% of cases in adult intussusceptions, and a primary or secondary malignant neoplasm causes about 40% of them. CT or ultrasonography showing a target sign or a pseudokidney sign usually helps in the diagnosis of intussusception. On colonoscopy, the finding of narrowing of the obstructive colonic lumen with smoothly oedematous bulging mucosa is suggestive of intussusception. As the disease progresses, the venous flow of the intussusceptum is impeded, followed by arterial supply. The intussusceptum may appear congested, friable, ulcerated or haemorrhagic on colonoscopy. The endoscopic differential diagnosis in this case should include intramural haematoma, and vascular tumours such as haemangioma, or angiosarcoma.

doi: 10.1136/gut.2006.096305