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Optimizing neurogenic potential of enteric neurospheres for treatment of neurointestinal diseases

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ABSTRACT

Background: Enteric neurospheres derived from postnatal intestine represent a promising avenue for cell replacement therapy to treat Hirschsprung disease and other neurointestinal diseases. We describe a simple method to improve the neuronal yield of spontaneously formed gut-derived neurospheres.

Materials and methods: Enteric neurospheres were formed from the small and large intestines of mouse and human subjects. Neurosphere size, neural crest cell content, cell migration, neuronal differentiation, and neuronal proliferation in culture were analyzed. The effect of supplemental neurotrophic factors, including glial cell line-derived neurotrophic factor (GDNF) and endothelin-3, was also assessed.

Results: Mouse small intestine–derived neurospheres contained significantly more P75-expressing neural crest-derived cells (49.9 ± 15.3% *versus* 21.6 ± 11.9%, $P < 0.05$) and gave rise to significantly more Tuj1-expressing neurons than colon-derived neurospheres (69.9 ± 8.6% *versus* 46.2 ± 15.6%, $P < 0.05$). A similar pattern was seen in neurospheres isolated from human small and large intestine (32.6 ± 17.5% *versus* 10.2 ± 8.2% neural crest cells, $P < 0.05$; 29.7 ± 16.4% *versus* 16.0 ± 13.5% enteric neurons, $P < 0.05$). The addition of GDNF to the culture media further improved the neurogenic potential of small intestinal neurospheres (75.9 ± 4.0% *versus* 67.8 ± 5.8%, $P < 0.05$) whereas endothelin-3 had no effect.

Conclusions: Enteric neurospheres formed from small intestine and supplemented with GDNF yield an enriched population of neural crest-derived progenitor cells and give rise to a high density of enteric neurons.

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Introduction

The enteric nervous system (ENS) is a complex network of neurons and glia which controls many essential functions of the gastrointestinal tract.[1] Diseases of the ENS encompass a broad spectrum of common gastrointestinal disorders including esophageal achalasia, gastroparesis, slow-transit constipation, and Hirschsprung disease.[2] Current treatment for these diseases is palliative rather than curative, although cell therapy holds promise as a novel potential therapy for this group of diseases.[3] Recent evidence has shown that enteric neural stem cells (ENSCs) can be isolated from the adult gut and propagated in culture as enteric neurospheres[4] and that these neurospheres can be transplanted to aneural gut, where they give rise to new neurons that may improve bowel function.[5]

However, in addition to neural and glial progenitors, enteric neurospheres also contain many other, nonneuroglial cell types.[6] Many techniques have been used to improve the neurogenic potential of enteric neurospheres. We have previously shown that co-transplantation of neurospheres with a serotonin receptor agonist can enhance neuronal differentiation and proliferation.[7] Modified dissection techniques to isolate the enteric nerve plexuses from donor tissue have been described to generate a purer population.[8] Cell sorting using neural crest cell markers have also been used to enrich the progenitor population within neurospheres,[6] but this would be difficult to apply clinically and may eliminate bystander cells important to supporting neuronal growth.

Previous reports have noted that the small intestine (SI) contains more than double the number of myenteric neurons than the large intestine (LI),[9] likely owing to the longer length of the SI. The length and redundancy of the SI make it an attractive tissue source, but neurospheres derived from SI and LI have not been compared. Other factors, such as glial cell line-derived neurotrophic factor (GDNF) and endothelin-3 (ET3), are both known to play an important role in ENS development, but their effect on postnatal gut-derived neurospheres has not been explored. ET3, through its receptor endothelin receptor type B (EDNRB), promotes ENSC proliferation, whereas GDNF, through the receptor tyrosine kinase, rearranged during transfection (RET), promotes ENSC migration, proliferation, and neuronal differentiation.[10] Mutations in either *RET/GDNF* or *EDNRB/ET3* pathway cause Hirschsprung disease in mice and humans.[11]

The goal of this study was to identify the optimal source for ENSCs and to explore methods to optimize their neurogenic potential. Our study identifies clinically useful observations for the use of neuronal cell therapy for the treatment of enteric neuropathies.

Materials and methods

Generation of mouse enteric neurospheres

With approval from the Institutional Animal Care and Use Committee, neurospheres were generated from male and female 3-wk old C57BL/6 mice (Jackson Labs, Bar Harbor, ME) according to previously published protocols.[7,12] The muscularis propria, which contains the myenteric plexus, was isolated from SI (duodenum through ileum; Fig. 1A and B) and LI (postcecal colon through anus, Fig. 1D and E). Primary neurospheres were dissociated with Accutase (Stemcell Technologies, Vancouver, BC) at 37°C for 30 min and replated at 50,000 cells/mL to form secondary neurospheres (Fig. 1C and F).

Generation of human enteric neurospheres

With approval from the institutional review board, 1-3 cm^2 pieces of SI or LI tissue were obtained from five patients (age: 1 mo-21 y) undergoing bowel resection, including SI and LI tissue from a 17-year-old male undergoing ileocecal resection (Table). Neurospheres were generated based on previously published protocols.[4,13] In brief, the muscularis propria was isolated and digested for 90 min at 37°C in dispase (250 μg/mL; Stemcell Technologies) and collagenase XI (1 mg/mL; Sigma-Aldrich, St. Louis, MO) and then filtered through a 70 μm filter. Cells were cultured in a 1:1 mix of mouse conditioned media (obtained from the supernatant of cultured mouse neurospheres) and human proliferation media, consisting of NeuroCult Human Basal Medium (Stemcell Technologies) supplemented with 10% NeuroCult Human Proliferation Supplement (Stemcell Technologies), 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor, 0.0002% Heparin, 50 μg/mL metronidazole (Sigma-Aldrich), 2 μL/mL Primocin (Invitrogen, Carlsbad, CA). After 7 d, primary neurospheres were dissociated with Accutase at 37°C for 30 min and replated at 50,000 cells/mL in a 96-well round bottom plate (Corning, Kennebunk, ME), which was centrifuged at 480 *g* for 2 min to encourage cell aggregation. Secondary neurospheres formed after 7 d in culture.

Tissue preparation and immunohistochemistry

Tissue preparation and immunohistochemistry were performed as previously described.[7] Cells and tissues were fixed in 4% paraformaldehyde. For cryosection, sections were cut at 12 μm thickness with a Leica CM3050 S cryostat (Leica, Buffalo Grove, IL). For immunohistochemistry, cells and tissues were permeabilized with 0.1% Triton X-100 and blocked with 10% donkey serum for 30 min. Primary antibodies included human antineuronal nuclear antibody-1 (Hu; 1:16,000; generous gift from Dr Vanda Lennon), mouse antineuronal class III β-tubulin (Tuj1; 1:500; Covance, Dedham, MA), rabbit anti-p75 neurotrophin receptor (P75; 1:500; Promega, Madison, WI), rabbit anti-S100 calcium-binding protein B (S100; 1:100; NeoMarkers, Fremont, CA), and rabbit anti-α-smooth muscle actin (1:100; Abcam, Cambridge, MA). Secondary antibodies included donkey antimouse Alexa Fluor 488, donkey antirabbit Alexa Fluor 546, and donkey antihuman Alexa Fluor 546 (Life Technologies, Carlsbad, CA). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). EdU incorporation was detected using the Click-iT EdU Imaging Kit (Invitrogen). Images were taken using a Nikon Eclipse TS100 or 80i microscope (Nikon Instruments, Melville, NY).