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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Enteric Neural Cells From Hirschsprung Disease Patients Form Ganglia in Autologous Aneuronal Colon

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SUMMARY

We show that enteric neural cells isolated from Hirschsprung disease patients can colonize aneuronal colon tissue to generate neurons and glia. Our findings establish the therapeutic potential of using patient's own neural cells to form an enteric nervous system in autologous tissue.

BACKGROUND & AIMS: Hirschsprung disease (HSCR) is caused by failure of cells derived from the neural crest (NC) to colonize the distal bowel in early embryogenesis, resulting in absence of the enteric nervous system (ENS) and failure of intestinal transit postnatally. Treatment is by distal bowel resection, but neural cell replacement may be an alternative. We tested whether aneuronal (aganglionic) colon tissue from patients may be colonized by autologous ENS-derived cells.

METHODS: Cells were obtained and cryopreserved from 31 HSCR patients from the proximal resection margin of colon, and ENS cells were isolated using flow cytometry for the NC marker p75 (nine patients). Aneuronal colon tissue was obtained from the distal resection margin (23 patients). ENS cells were assessed for NC markers immunohistologically and by quantitative reverse-transcription polymerase chain reaction, and mitosis was detected by ethynyl-2′-deoxyuridine labeling. The ability of human HSCR postnatal ENS-derived cells to colonize the embryonic intestine was demonstrated by organ coculture with avian embryo gut, and the ability of human postnatal HSCR aneuronal colon muscle to support ENS formation was tested by organ coculture with embryonic mouse ENS cells. Finally, the ability of HSCR patient ENS cells to colonize autologous aneuronal colon muscle tissue was assessed.

RESULTS: ENS-derived p75-sorted cells from patients expressed multiple NC progenitor and differentiation markers and proliferated in culture under conditions simulating Wnt signaling. In organ culture, patient ENS cells migrated appropriately in aneural quail embryo gut, and mouse embryo ENS cells rapidly spread, differentiated, and extended axons in patient aneuronal colon muscle tissue. Postnatal ENS cells derived from HSCR patients colonized autologous aneuronal colon tissue in cocultures, proliferating and differentiating as neurons and glia.

CONCLUSIONS: NC-lineage cells can be obtained from HSCR patient colon and can form ENS-like structures in aneuronal colonic muscle from the same patient. *(Cell Mol Gastroenterol Hepatol 2016;2:92–109; http://dx.doi.org/10.1016/j.jcmgh.2015.09.007)*

Keywords: Hirschsprung Disease; Aganglionosis; Cell Therapy; Megacolon; Enteric Nervous System.

The enteric nervous system (ENS) is a huge ganglionated network that coordinates bowel motility.[1] Hirschsprung disease (HSCR) is a congenital condition in which the ENS is absent from the distal bowel.[2] This aneuronal (often termed aganglionic) bowel segment prevents passage of intestinal contents and causes distension (megacolon) with potentially fatal outcome. Treatment for HSCR is surgical resection of the aganglionic bowel and anastomosis of the neuronal (ganglionated) proximal bowel to the anorectum, but constipation and fecal soiling are common postoperative complications.[3] Cell-based therapies have been proposed to repopulate the aneuronal bowel by the transplantation of stem or progenitor cells.[4–6]

The ENS is derived from embryonic neural crest (NC) cells, mostly from the vagal level of the neural tube.[7] Vagal NC cells enter the proximal gastrointestinal tract and as

Abbreviations used in this paper: **CHIR-99021, 6-[2-[[4-(2,4-dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl)pyrimidin-2-yl]amino] ethylamino]pyridine-3-carbonitrile; EdU, ethynyl-2′-deoxyuridine; eGFP, enhanced green fluorescent protein; ENC, enteric neural crest; ENS, enteric nervous system; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GSK3, glycogen synthase kinase 3; HNK1, human natural killer-1; HSCR, Hirschsprung disease; MTR, MitoTracker Red; NC, neural crest; nNOS, neuronal nitric oxide synthase; nTCM, neural tissue culture medium; PBS, phosphate-buffered saline; PFA, paraformaldehyde; qRT-PCR, quantitative reverse transcription and polymerase chain reaction; RCH, Royal Children's Hospital; SMA, smooth muscle actin; SOX10, sex-determining region Y–box 10; TUJ1, neuron-specific class III β-tubulin.**

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2352-345X
http://dx.doi.org/10.1016/j.jcmgh.2015.09.007

enteric neural crest (ENC) cells migrate along the entire tract in a distal-directed wave over the 4th to 7th weeks of gestation in humans.[8–10] Experiments in animal models indicate that the sacral level of the NC also provides neurons to the gut,[11,12] but these are numerically less capable[13] and cannot compensate for a lack of vagal NC-derived cells.[14,15] Recently a late-arising population of ENS neurons has been identified in mice derived from Schwann cell intermediaries; these are a minority source and give a restricted range of neuron types.[16] HSCR results when this wave does not complete colonization of the entire gut. Cell-replacement therapy postnatally after HSCR diagnosis for a defect of early embryonic origin faces difficulties. ENC stem or progenitor cells would have to be obtained and expanded before transplantation; then they would have to be introduced into the aneuronal gut without immune rejection. They must then migrate, colonize, differentiate, and establish connections to mediate bowel function at later stages and in a far larger tissue than would occur during normal ENS development.

Rodent and avian embryonic ENC progenitor cells can colonize embryonic aneural gut and form ENS in vitro.[17–20] Further, rodent embryo NC cells can colonize the aneuronal gut and differentiate into neurons and glia of postnatal HSCR model mice in vivo.[21,22] In addition, it has been shown that postnatal mouse ENC cells surgically placed in the wall of the colon of postnatal normal and HSCR-model syngeneic mice are able to migrate, proliferate, assemble into correctly placed ganglia and differentiate as both glia and neurons that extend axons, make and receive synapses, and show electrical activity.[23] This is a key step to answering the therapeutic questions of whether ENS formation can be accomplished when both ENS-lineage donors and colon tissue recipients are postnatal. A further question is, can this be accomplished when both ENC and colon are not only of postnatal human origin, but when (to avoid immune rejection) they are of autologous HSCR patient origin?

We obtained human ENS-derived cells from the proximal margin of HSCR patient colon using flow cytometry for the NC marker p75. These cells were assessed for additional NC markers by antibody labeling and quantitative reverse-transcription polymerase chain reaction (qRT-PCR), and for proliferation with ethynyl-2′-deoxyuridine (EdU). We tested the ability of these cells to migrate in embryonic aneuronal gut in organ culture by combining the human ENS-derived cells with quail embryo intestine,[24] a tissue known to support cell colonization. We then ascertained the ability of human postnatal HSCR aneuronal distal colon muscle tissue to support ENS formation by, in organ culture, transplanting into it genetically labeled ENC-derived cells from embryonic mice, cells with proven ENS-forming ability.[23] We then labeled human p75-sorted cells using fluorescent tags and implanted them into distal colonic muscle tissue from the same HSCR patients and maintained the combinations in organ culture. The results show that NC-lineage ENS cells can be isolated from neuronal regions of HSCR patient postnatal colon, and that these ENS-derived cells can spread, proliferate, and differentiate in receptive intestinal tissues including aneuronal HSCR colon muscle from the same patient.

Materials and Methods

Human Tissues

Colon tissue was obtained immediately after surgery for HSCR at the Royal Children's Hospital (RCH). In most cases (24 of 31), the patients were younger than 4 months at surgery, the length of resected bowel was less than 12 cm (27 of 31), and no other clinical conditions were identified (Table 1). Familial forms of HSCR (patients with first- and second-degree relatives affected) were reported in a small number of cases (4 of 31). After obtaining tissue from the proximal (neuronal) and distal (aneuronal) ends, the remainder of resected bowel tissue was histologically examined at RCH Anatomical Pathology. Samples of the muscle layers of both ends of all specimens were fixed in 4% paraformaldehyde (PFA), embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura Finetek Europe, Alphen aan den Rijn, Netherlands), frozen, and sectioned (CM1900 cryostat; Leica Microsystems, Wetzlar, Germany) for immunolabeling. The procedures for resected tissues are described here, and a flow chart is shown in Figure 1.

Human tissue was collected under approval from the Royal Children's Hospital (Parkville, Melbourne, Australia) Human Research Ethics Committee (HREC 30014A). Parent/guardian consent was obtained for all participants. Animal tissues were obtained under the conditions of the Murdoch Children's Research Institute Animal Ethics Committee (AEC651).

Human Colon Tissue Dissociation

To obtain human ENS cells from myenteric plexuses between proximal colon muscle layers, the mucosa was removed by scraping, and the muscle layer was cut into 1–2 mm^3 fragments. Dissociation was achieved in Ham's F-12 media (GIBCO/Invitrogen, Grand Island, NY) supplemented with 0.5% w/v Dispase II (Roche, Basel, Switzerland) and

Table 1. Source of Human Gut Samples: Demographic and Clinical Characteristics of Children With Hirschsprung Disease and Length of the Colon-Rectum Resected During the Surgical Procedure

Type of Surgical Procedure	No. of Children (n = 31)	Age at Surgical Procedure (mo)	Length of Colon-Rectum Resected (cm)
Laparoscopic-assisted and transanal pull-through	16	3.7 ± 2.2	15.4 ± 7.5
Transanal endorectal pull-through (de La Torre-Soave)	10	4.0 ± 2.0	11.8 ± 5.2
Duhamel	3	24 ± 22.3	Total colonic
Swenson	1	169	67
Colectomy (Re-do)	1	102	11

Note: Age and length values are mean ± standard deviation.