Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

Increased population of immature enteric glial cells in the resected proximal ganglionic bowel of Hirschsprung's disease patients

Gakuto Tani, MD,[a] *Christian Tomuschat, MD,*[a] *Anne Marie O'Donnell, PhD,*[a] *David Coyle, MD,*[a] *and Prem Puri, MS, FRCS*[a,b,*]

[a] *National Children's Research Centre, Our Lady's Children's Hospital, Crumlin, Dublin, Ireland*

[b] *School of Medicine and Medical Science and Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Ireland*

ARTICLE INFO

Article history:
Received 28 December 2016
Received in revised form
9 March 2017
Accepted 18 May 2017

Keywords:
Hirschsprung's disease
Enteric glial cells
Fatty acid–binding protein 7
Premature glia

ABSTRACT

Background: Enteric glial cells are essential for normal gastrointestinal function. Abnormalities in glial structure, development, or function lead to disturbances in gastrointestinal physiology. Fatty acid–binding protein 7 (FABP7) is a marker of immature enteric glial cells, whereas S100 is expressed only by mature glial cells. Patients with Hirschsprung's disease (HSCR) often suffer from dysmotility and enterocolitis despite proper surgery. We designed this study to determine the distribution and expression of glial cells in patients with HSCR compared to normal controls.

Methods: We investigated FABP7, S100, and PGP 9.5 expressions in both the ganglionic and aganglionic bowel of patients with HSCR ($n = 6$) *versus* normal control colon ($n = 6$). Protein distribution was assessed by using immunofluorescence and confocal microscopy. Gene and protein expressions were quantified using quantitative real-time polymerase chain reaction (qPCR), Western blot analysis, and densitometry.

Results: qPCR and Western blot analysis demonstrated a significantly increased FABP7 expression in ganglionic specimens compared to control specimen ($P < 0.05$). Confocal microscopy revealed FABP7^{+} glia cells lie under the colonic epithelium and in close apposition to enteric neurons in the ganglionic bowel.

Conclusions: The significantly increased number of immature enteric glial cells (EGCs) in the ganglionic bowel of HSCR patients may have adverse effect on the function of enteric neurons and intestinal barrier and thus predispose these patients to intestinal motility problems and enterocolitis.

Introduction

Hirschsprung's Disease (HSCR) is a developmental disorder of the enteric nervous system (ENS) characterized by the absence of ganglion cells in the distal bowel.[1] The lack of ganglion cells in HSCR is attributed to a failure of migration of neural crest cells. The earlier the arrest of migration, the longer the aganglionic segment.[2] The pathophysiology of HSCR is not

* *Corresponding author*. National Children's Research Centre, Our Lady's Children's Hospital, Crumlin, Dublin 12, Ireland. Tel.: +353 14096420; fax: +353 1455 201.

E-mail address: prem.puri@ncrc.ie (P. Puri).

0022-4804/$ – see front matter © 2017 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jss.2017.05.062

fully understood. Treatment of HSCR consists of the following: (1) to resect the aganglionic bowel, (2) to pull-down normoganglionic bowel, and (3) to perform a proper coloanal anastomosis.[3] However, some patients continue to have persistent bowel symptoms such as constipation, soiling, or enterocolitis after surgery.[4]

Recent studies have reported histopathological alterations in the ganglionic bowel, suggesting that the pulled-through ganglionic bowel in HSCR in not normal and may be responsible for persistent dysmotility problems and enterocolitis.[5-7]

The ENS is composed of enteric neurons and enteric glia. Enteric glial cells (EGCs) are located around the myenteric and submucosal plexuses and in the mucosa. In the mucosa, EGC lie below the epithelium and form extensive networks among the enteric nerves, endocrine cells, and immune cells. In the past, EGCs were considered to be nerve-supporting cells. However, it is now increasingly recognized that these cells play key roles in gastrointestinal motility and intestinal epithelial barrier function. All glial cells originate from neural crest progenitor cells. Most stem from an early wave of crest cells that migrate throughout the gastrointestinal tract giving rise to both neurons and glia.[8] The process of differentiation and maturation of enteric glial cells are tightly regulated, and failure of maturation is linked to numerous alterations in gut homeostasis.[8,9]

Glutamine synthetase (GS) and S100 are reported to be specific markers for mature enteric glial cells. Fatty acid–binding protein 7 (FABP7) is considered a marker of mainly immature glial cells.[8] In fact, EGC precursors are identified by brain fatty acid–binding protein and can be detected within 24 hr after gut segments have been colonized by neural crest cells.

In the light of the important role enteric glia cells play in gut homeostasis and disease, this study has been carried out to investigate the expression and distribution of immature EGC in the bowel of patients with HSCR.

Materials and methods

Tissue samples

This study was approved by the Ethics Medical Research Committee, Our Lady's Children's Hospital (Ref GEN.292/12), and tissue samples were obtained with informed parenteral consent. Study material included HSCR specimens from six patients and control specimens from six patients. HSCR specimens were divided into aganglionic and ganglionic samples. Ganglionic samples were taken from the most proximal margin of the pull-through specimen. Only specimens with ganglion cells in the submucosal and myenteric plexus have been taken for evaluation. Furthermore, the specimens picked for further assessment; nerve fiber diameter is measured to ensure that specimens contain no hypertrophic nerve bundles (<40 μm). Normal control samples were taken from patients who underwent sigmoid colostomy closure following anorectoplasty for imperforate anus. Tissue specimens were either snap-frozen in liquid nitrogen and stored at −80°C for protein extraction or embedded in OCT Mounting Compound (VWR International, Leuven, Belgium) for immunofluorescence and stored at −80°C until use.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA from the myenteric plexus was extracted to examine FABP7, S100, and PGP 9.5 expressions in the ganglionic bowel and controls. Frozen tissue was sectioned with the cryostat, and the sections were processed for hematoxylin staining. After the sections had been stained, myenteric plexus were dissected by laser capture microdissection (ArcturusXT Laser Capture Microdissection System, MDS Analytical Technologies). RNA was extracted from dissected myenteric plexus according to the procedure of PicoPure RNA Isolation Kit (Arcturus, UK), and complementary DNA was synthesized from extracted RNA according to the procedure of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR was performed by SYBR Green I assay (LightCycler 480 SYBR Green I Master Mix, Roche Diagnostics, Mannheim, Germany). Gene-specific primer pairs are showed as follows. FABP7 primer sequences were 5′-TCT CTG TGA CAG CCT CTT GG-3′ (forward) and 5′-GCG GGG ATC TTC TGGTCT AA-3′ (reverse). S100 primer sequences were 5′-AGG ATG CCG TGG ATA AAT TG-3′ (forward) and 5′-GTG ACA GGC AGA CGT GAT TG-3′ (reverse). PGP 9.5 primer sequences were 5′-AAC AAT CAG CAA TGC CTG TG-3′ (forward) and 5′-TCC AGG TAT CTG GCT CGT TC-3′ (reverse). Glyceraldehyde-3-phosphate dehydrogenase primer sequences were 5′-CAC CAC ACT GAA TCT CCC CT-3′ (forward) and 5′-TGG TTG AGC ACA GGG TAC TT-3′ (reverse). After initial denaturation at 95°C for 5 minutes, 55 cycles of amplification for each primer were carried out. Each cycle included denaturation at 95°C for 10 seconds, annealing at 60°C for 15 seconds, and elongation at 72°C for 10 seconds. Each messenger RNA (mRNA) expression was normalized against the glyceraldehyde-3-phosphate dehydrogenase expression.

Immunofluorescence staining and confocal microscopy

Frozen blocks of HSCR colon and normal control samples were sectioned transversely at a thickness of 10 μm, mounted on Superfrost Plus slides (VWR International, Leuven, Belgium), and fixed with buffered 10% formalin for 10 min. Sections underwent cell membrane permeabilization with 1% TritonX-100 for 25 min at room temperature. After blocking with 5% bovine serum albumin for 30 min to avoid nonspecific absorption, sections were incubated with primary antibodies: FABP7 (1:200, ABN14, Millipore), GS (1:200, sc-74430, Santa Cruz Biotechnology, CA), and S100 (1:200, ab11178, Abcam, Cambridge) overnight at 4°C. Sections were then washed in PBS-0.05% Tween and incubated with corresponding secondary antibodies (1:1000, anti-mouse IgG AlexaFluor&reg 594; ab150116, anti-mouse IgG AlexaFluor 647; ab150103, anti-rabbit IgG AlexaFluor 488; ab150073, or anti-goat IgG AlexaFluor 555; ab150130, Abcam, Cambridge) for 1 hr at room temperature. After washing, sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole) antibody, dilution 1:1000 (Roche Diagnostics GmbH, Mannheim, Germany) for 15 min, washed, mounted, and coverslipped with Fluorescent Mounting Medium (DAKO Ltd, Cambridgeshire, UK). All