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Bowel dysfunction following pullthrough surgery is associated with an overabundance of nitrergic neurons in Hirschsprung disease

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ABSTRACT

Purpose: Recent evidence suggests that patients with Hirschsprung disease (HD) have abnormal neurotransmitter expression in the ganglionated proximal colon. These alterations may cause persistent bowel dysfunction even after pullthrough surgery. We sought to quantify the proportion of nitrergic neurons in the ganglionic colon of HD patients and relate these findings to functional outcome.
Methods: The proximal resection margin from 17 patients with colonic HD who underwent a pullthrough procedure and colorectal tissue from 4 age-matched controls were immunohistochemically examined to quantify the proportion of nitrergic neurons. The incidence of constipation, incontinence, and enterocolitis in HD patients was assessed retrospectively and correlated with the proportion of nitric oxide synthase (NOS) expressing neurons. Neuronal subtypes in the ganglionic colon of the *Ednrb* $^{-/-}$ mouse model of HD were also studied.
Results: Mice with HD had a significantly higher proportion of NOS+ neurons in ganglionic colon than normal littermates (32.0 ± 5.6% vs. 19.8 ± 1.2%, $p < 0.01$). Patients with HD also had significantly more NOS+ neurons than controls (18.4 ± 4.6% vs. 13.1 ± 1.9%, $p < 0.01$). Patients who experienced constipation or enterocolitis postoperatively tended toward a higher proportion of NOS+ neurons (21.4 ± 3.9% vs. 17.1 ± 4.1%, $p = 0.06$). Furthermore, patients with a proportion of NOS+ neurons above the median of all HD patients (18.3%) were significantly more likely to have constipation than those below the median (75% vs. 14%, $p < 0.05$).
Conclusion: An overabundance of nitrergic neurons in the proximal resection margin is associated with HD and may predict bowel dysfunction following pullthrough surgery.

Hirschsprung disease (HD) is a common congenital disease characterized by the absence of enteric ganglia in the distal intestine [1]. Even after pullthrough surgery, patients often suffer from persistent problems with constipation, fecal incontinence, and Hirschsprung-associated enterocolitis (HAEC) [2]. Though functional outcome may improve with age, long-term studies have found that many patients with HD continue to have impaired bowel function and, as a result, suffer psychosocial stress into adulthood [3,4].

Recent studies have suggested that the remaining ganglionic intestine in HD patients may not be normally innervated and that these latent abnormalities could be responsible for persistent bowel dysfunction in some patients [5]. Abnormalities in neurotransmitter expression have been described in the ganglionic bowel of HD patients, with a relative overabundance of nitrergic innervation and a deficit of cholinergic innervation [6]. The overexpression of nitric oxide synthase (NOS) and its related proteins is unique to HD and are not a result of bowel dilatation alone [7]. Similar alterations in NOS expression have been observed in several mouse models of HD and are linked to intestinal dysmotility [8–10]. Intestinal motility relies on a careful balance between inhibitory (e.g. nitrergic) and excitatory (e.g. cholinergic) innervation, and any perturbation of that balance could lead to dysmotility [11–13].

Though it has been suggested that these abnormalities may cause postoperative bowel dysfunction, no studies have yet examined the association between functional outcomes and NOS expression in HD. In addition, the proportion of nitrergic neurons in the proximal resection margin of HD patients has not been quantified immunohistochemically, and this is necessary to establish the overabundance of NOS+ neurons as a pathological marker of disease. In this study, we sought to quantify the proportion of nitrergic neurons in ganglionic colon of mice and children with HD and correlate these abnormalities with functional outcome after pullthrough in children.

1. Materials and methods

1.1. Animal subjects

With approval from the Institutional Animal Care and Use Committee, tissue was obtained from 3-week old homozygous *Ednrb*^{tm1Ywa}

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(*Ednrb*$^{-/-}$) mice on a hybrid C57BL6/J-129Sv background (B6;129-*Ednrb*$^{tm1Ywa/J}$; Jackson Labs) or their wild-type (*Ednrb*$^{+/+}$) littermates. *Ednrb*$^{-/-}$ mice, characterized by distal colonic aganglionosis and a piebald color, are a commonly used model for human HD [14]. After euthanasia, colons were removed, opened along the mesenteric border, and flushed of enteric contents. The whole colon was fixed overnight in 4% paraformaldehyde and tissue embedded in 15% sucrose containing 7.5% gelatin for cryosection.

1.2. Human subjects

With approval from the institutional review board, paraffin-embedded biopsies of the proximal resection margin were obtained from 17 patients with partial colonic HD who had undergone a pullthrough procedure (Soave) between September 2003 and April 2016 at a single institution. All resection margins had been examined by pathologists and considered to be normoganglionic. For comparison, colorectal biopsies were obtained from 4 age-matched control patients, including 2 patients with chronic constipation who had rectal biopsies to rule out HD, 1 patient with anorectal malformation (ARM), and 1 patient with a sigmoid stricture after necrotizing enterocolitis.

For patients with HD, charts were retrospectively reviewed to determine length of bowel resection and clinical outcome including need for reoperation, constipation, fecal incontinence, and HAEC. A senior surgical resident (D.M.S.) reviewed patient outcomes blinded to the immunohistochemical findings for each patient.

1.3. Immunohistochemistry

Immunohistochemical studies were performed on either 12 μm cryosections (mice) or 5 μm paraffin-embedded sections (humans). For cryosections, slides rehydrated in warmed PBS to remove surrounding gelatin. For paraffin-embedded tissue sections, slides were heated at 65 °C for 1 h, then rehydrated by sequentially soaking in xylene (5 min × 3), ethanol (100% for 5 min × 3, followed by 90%, 75%, 50%, 25%, 10% for 1 min each), water (5 min × 3), and phosphate-buffered saline (PBS; 5 min × 3). Antigen retrieval was performed by heating slides in a 1 M sodium citrate buffer solution (15 min).

For both cryosections and paraffin-embedded sections, tissue was permeabilized and blocked with 0.1% Triton X-100, 10% donkey serum, and 10% bovine serum albumin in PBS for 1 h. Primary antibodies were applied overnight for 12–16 h at 4 °C and secondary antibodies were applied for 1 h at room temperature. Primary antibodies included: mouse anti-neuronal class III β-tubulin (Tuj1, 1:500; Covance, Dedham, MA), rabbit anti-neuronal nitric oxide synthase 1 (NOS, 1:500; Santa Cruz Biotechnology, Dallas, TX), rabbit anti-calretinin (CR, 1:200; Invitrogen, Carlsbad, CA). Neuronal class III β-tubulin (Tuj1) marks all enteric neurons; calretinin (CR) immunoreactivity labels enteric, and not extrinsic, nerves [15,16]; and NOS immunoreactivity indicates nitrergic neurons in the gut. Secondary antibodies used were: donkey anti-mouse Alexa Fluor 488 (1:1000; Invitrogen) and donkey anti-rabbit Alexa Fluor 546 (1:1000; Life Technologies, Carlsbad, CA). Cell nuclei were stained with DAPI. Images were taken using a Nikon Eclipse 80i microscope. Three or more randomly-selected myenteric ganglia were imaged for each subject. Cell numbers were quantified using ImageJ software (NIH, Bethesda, MD).

1.4. Statistics

Data are presented as mean ± standard deviation. Results were compared using the Student's unpaired t-test for continuous variables and Fisher's exact test for dichotomous variables. Statistical significance was considered at $p < 0.05$. Statistical analysis was performed using Graphpad Prism version 7.0 for Windows (Graphpad Software, La Jolla, CA).

2. Results

2.1. *Ednrb*$^{-/-}$ mice have a greater proportion of nitrergic neurons in the ganglionic colon than *Ednrb*$^{+/+}$ littermates

In the aganglionic distal colon of *Edrnb*$^{-/-}$ mice, hypertrophic extrinsic nerves were immunoreactive for Tuj1, but negative for CR (Fig. 1A) and NOS (Fig. 1D), distinguishing them from intrinsic ganglia. In the transition zone, small ganglia consisting of CR + Tuj1 + (Fig. 1B) and NOS + Tuj1 + (Fig. 1E) cells were present along with scant NOS + Tuj1 + and CR + Tuj1 + double-positive fibers in the circular muscle. Proximal to the transition zone, we observed normal-appearing ganglia immunoreactive for CR (Fig. 1C) and NOS (Fig. 1F), along with abundant NOS + Tuj1 + and CR + Tuj1 + fibers throughout the muscularis propria.

The presence of NOS + Tuj1 + and CR + Tuj1 + neurons in the myenteric ganglia of ganglionated colon, approximately 1–2 cm proximal to the transition zone, was analyzed quantitatively. The proportion of CR + Tuj1 + neurons in the ganglionic segment did not differ significantly in *Ednrb*$^{-/-}$ mice and *Ednrb*$^{+/+}$ littermates (19.2 ± 5.8% vs. 25.1 ± 4.3%, $p = 0.15$; Fig. 1G). However, the ganglionic colon of *Ednrb*$^{-/-}$ mice had a greater proportion of NOS + Tuj1 + neurons compared to the equivalent colonic segment of *Ednrb*$^{+/+}$ littermates (32.0 ± 5.6% vs. 19.8 ± 1.2%, $p < 0.01$; Fig. 1H). The ratio of NOS + Tuj1 + to CR + Tuj1 + neurons in *Ednrb*$^{-/-}$ mice was also significantly greater than in *Ednrb*$^{+/+}$ littermates (1.8 ± 0.6% vs. 0.8 ± 0.2; $p < 0.05$; Fig. 1I).

2.2. Children with HD have a greater proportion of nitrergic neurons in the ganglionic colorectum than control patients

Patients with HD ranged in age from 9 days old to 7 months old (median = 2 months old) at the time of pullthrough, 88% were male, and 12% had Down syndrome. In comparison, control patients ranged in age from 1 to 9 months old (median = 3 months old), 100% were male, and none had Down syndrome. In colorectal samples from the ganglionic segment of HD patients and controls, all ganglia exhibited immunoreactivity for CR (Fig. 2D and E). In contrast, hypertrophied extrinsic nerves from the distal aganglionic segments of HD patients were negative for CR (Fig. 2B-C). All ganglia were also immunoreactive for NOS (Fig. 2H-I), whereas extrinsic nerves were not (Fig. 2F-G). No hypertrophied nerves were seen in specimens obtained from the proximal resection margin.

We found no statistical difference in the proportion of CR + Tuj1 + neurons in the ganglionic segment (Fig. 3A and B) compared to controls (8.7 ± 3.4% vs. 8.5 ± 1.7%, $p = 0.82$; Fig. 3E). In contrast, the proportion of NOS + Tuj1 + enteric ganglion cells in HD patients (Fig. 3B and C) was significantly greater than controls (18.4 ± 4.6% vs. 13.1 ± 1.9%, $p < 0.01$; Fig. 3F). In addition, the ratio of NOS + Tuj1 + to CR + Tuj1 + neurons was also greater in HD patients, although this did not reach statistical significance (2.4 ± 1.0 vs. 1.6 ± 0.7, $p = 0.11$; Fig. 3G).

2.3. Clinical outcomes in patients with HD

Of 17 patients, 13 had short-segment disease confined to the rectosigmoid and 4 patients had aganglionosis extending to the splenic flexure. All patients underwent bowel resection with transanal Soave endorectal pullthrough. The average length of colon resected was 16.3 ± 6.5 cm. The median length of follow-up was 6 years (range: 1 month to 12 years). Three patients (18%) required reoperation after pullthrough for incisional hernia, anastomotic stricture, and lysis of adhesions. No patient required reoperation for residual aganglionosis or transition zone pullthrough. Eight patients (47%) experienced constipation after pullthrough. Two patients with constipation were also diagnosed with anastomotic stricture; one was successfully treated with dilatation and the other required reoperation. Of the two patients