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A Durable Model of Hirschsprung's Colon

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Abstract

Introduction—Hirschsprung's disease is characterized by colonic aganglionosis, curable only by surgical correction. Stem cells may offer regenerative benefits while preventing surgical risks. Existing Hirschsprung's model systems are limited by alimentary compromise and spontaneous ganglionic reconstitution. We endeavored to generate a model of permanent colonic aganglionosis to support longitudinal cell therapy studies.

Methods—Among adult female Lewis rats (n=11), laparotomy was performed and one-centimeter segments of descending colon were isolated from continuity and denervated by trans-serosal benzalkonium chloride (BAC) exposure. Postoperative weights were plotted. The colon segments were retrieved after 50 or 100 days. Immunohistochemical staining (IHC) for beta-III tubulin (TUB1) and glial fibrillary acid protein (GFAP) revealed colonic ganglia. Muscle layer diameter and the presence of ganglia were contrasted between normal and denervated segments.

Results—All animals survived, experienced 5% weight loss after one week, and then consistently gained weight. Isolated segments had significantly hypertrophied smooth muscle layers compared to normal colon. Ganglia were identified by IHC in normal colonic segments, and denervated colonic segments had no IHC evidence of myenteric ganglia.

Conclusion—Colonic segmental isolation and denervation results in an effective model of irreversible colonic aganglionosis. Animals retain alimentary function. Muscularis hypertrophy, myenteric denervation, and normal animal longevity are suitable for long-term studies of cell therapy.

Keywords

Hirschsprung's disease; Aganglionosis; Regenerative medicine; Stem cell; Cell-based therapy; Aganglionosis

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Introduction

Hirschsprung's disease afflicts approximately 1 in 5,000 live births in the United States each year.^{1,2} The disease is characterized by absent enteric ganglia in a segment of the rectum and a variable length of colon. Aberrant neural crest cell migration is the hypothesized etiology of Hirschsprung's disease. Surgical correction is essential, and it often involves resection of aganglionic colon. Approximately 60% of pediatric patients undergoing colonic resection for Hirschsprung's disease develop complications, including fecal incontinence, enterocolitis, enterostomal complications, reoperation, and death.¹⁻¹⁰

To circumvent the risks of major surgery, cell-based regenerative therapy is under investigation. Recent studies have demonstrated that transplanted neural crest-derived cells populate aganglionic intestine.¹¹⁻¹⁷ In these studies, recipient aganglionosis was induced by a variety of mechanisms. Knockout models of *Ret*, *Ednrb*, *Edn3*, *Gdnf*, *Sox10*, and *Phox2b* genes produce several phenotypes that mimic the spectrum of Hirschsprung's disease.^{2,18-20} Surgical models have also been generated in which benzalkonium chloride (BAC) is used to ablate enteric ganglia in a controlled fashion.^{21,22} These models have undergone extensive characterization studies reliably demonstrating myenteric aganglionosis, aperistalsis, and preservation of smooth muscle contractile potential.²³⁻²⁹ Both systems are frequently used in current studies of Hirschsprung's disease modeling and novel therapies.

Existing experimental model systems of Hirschsprung's disease significantly limit studies of cell-based therapy. Genetic knockout animals and those with chemically denervated segments suffer alimentary failure over time.^{2,30} This phenomenon occurs as a result of aperistalsis in the denervated segment and distention in the proximal intestine. In most cases, the model animals die within weeks.^{12,14,21,22,31} Studies of regenerative therapy are limited by the model animal's reduced lifespan. A more durable model of Hirschsprung's disease is necessary to assess the effects of cell-based therapy over time.

We recently reported a novel surgical procedure to induce intestinal segmental aganglionosis. In this procedure, we isolated a jejunal segment from intestinal continuity, chemically denervated the segment with BAC, and restored intestinal continuity with a bypass jejunojejunostomy. This procedure generated durable segmental aganglionosis, and it has facilitated studies of stem cell transplantation.¹⁷ A colonic analog is necessary for these studies to investigate the results of cell therapy within the intended recipient tissue. In this study, we present for the first time a model of Hirschsprung's colon that preserves the animal's alimentary function, and that may be used for time-intensive studies of stem cell therapy.

Materials and Methods

Animal Subjects

The following was conducted with the approval of our Institutional Animal Care and Use Committee under protocol #2006-061. Adult female Lewis rats (n=11) were obtained from Charles River Laboratories (Wilmington, MA). The average rat weight upon arrival was 253

g. Animals were housed in a United States Department of Agriculture-approved facility under veterinary observation for the duration of this study.

Operative Procedure

The operative procedure steps are illustrated in Figure 1. Animal subjects underwent midline laparotomy under inhaled isoflurane anesthesia. The descending colon was transected twice to isolate a 1-cm discontinuous segment approximately 4-5 cm cephalad to the anal verge. The mesocolon containing the inferior mesenteric artery was preserved. The isolated colonic segment was covered with gauze soaked with benzalkonium chloride (BAC, 0.2% w/v, Sigma-Aldrich) solution for 20 minutes. The ends of the isolated segment were closed and an end-to-end bypass colocolostomy was performed with 6-0 polypropylene suture to restore colonic continuity. The BAC-treated segment was irrigated with 0.9% normal saline and omentum was placed between the continuous and isolated colon. The abdominal fascia was reapproximated with 3-0 polyglactin, and the skin closed with 3-0 nylon. Trimethoprim sulfa (TMS, 1% v/v) was administered postoperatively for 14 days. The animals received a standard solid feeding regimen.

Postoperative Evaluation

Rat weights were measured twice weekly for the first 4 weeks, and then once weekly thereafter. The animals were sacrificed 50 or 100 days after colonic denervation. The isolated segment and a segment of normal descending colon were excised from each rat and fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA) for 24 hours at 4°C. Muscularis propria thickness was measured on cross-sectional slides stained with hematoxylin and eosin (H&E). A comparison between normal and denervated colon specimens was performed using unpaired T-tests.

Immunohistochemistry

Fixed colon specimens were embedded in paraffin wax and cut into 5- μ m sections. Paraffin was dissolved in xylenes for 10 minutes. Subsequently, slides were washed for 2 minutes serially in 100%, 95%, and 70% ethanol (Fisher Scientific) followed by water. The sections were incubated for 20 minutes at 95°C in citrate buffer (Biogenex, San Ramon, CA) for antigen retrieval, then cooled for 30 minutes in a water bath. A hydrophobic PAP pen (Vector Laboratories, Burlingame, CA) was used to generate a barrier around each section. Slides were exposed to a blocking solution of 5% normal goat serum (Vector Laboratories) and 2% bovine serum albumin in PBS with 0.05% Tween-20 (PBS/T) for one hour at room temperature.

Primary antibody suspensions were then applied to the blocked slides and incubated in a humidified slide chamber at 4°C overnight. Primary antibodies were anti-neuron specific β -III-tubulin (TUJ1; 5 μ g/ml; Abcam) and anti-glial fibrillary acid protein (GFAP; 1:400; Sigma-Aldrich). After incubation, the slides were washed three times for 30 minutes in PBS/T. Secondary antibodies were diluted in PBS/T and exposed to the tissue sections for 30 minutes at room temperature. The secondary antibody was goat anti-mouse Alexa Fluor® 594 (all 1:200; Invitrogen). We then performed three final washes with PBS/T. Each section was treated with Prolong Gold with DAPI (Invitrogen) and covered with a glass coverslip.

Histological slides were visualized under fluorescence microscopy (Leica Microsystems, Bannockburn, IL) to contrast the presence of ganglia among normal and denervated colon specimens.

Results

All rats successfully underwent colonic segment isolation and chemical denervation, and all survived to planned euthanasia. Postoperative colonic anatomy is shown in Figure 2.A. As expected, the denervated segment was grossly distended, and the colon proximal and distal to the bypass anastomosis was of normal caliber. Animals lost an average of 4.5% of their baseline body weights within 2 days of the denervation procedure. All animals recovered their preoperative weights within 13-16 days and continued to gain weight at a mean rate of 0.33% body weight daily (Figure 2.B).

The denervation procedure resulted in hypertrophy of the colonic muscularis propria (Figure 3). Overall muscularis thickness was significantly greater in the BAC-treated isolated tissue specimens than in normal colon (219 ± 9 vs. 114 ± 11 μm ; $P < 0.001$). When parsed by layer, these differences were evident in both the longitudinal (44 ± 5 vs. 30 ± 5 μm ; $P < 0.001$) and circular (175 ± 7 vs. 84 ± 9 μm ; $P < 0.001$) smooth muscle.

IHC evaluation demonstrated obliteration of ganglia after chemical denervation, as expected (Figure 4.C-F). When quantified per cross-section under magnification, approximately 90% of TUJ1-expressing myenteric cell clusters were ablated in the denervated segments (Figure 4.G-H). This effect was evident at all post-denervation time points up to 100 days.

Discussion

Our procedure results in durable colonic aganglionosis. IHC analysis demonstrated ablation of myenteric neuronal and glial cells that persists at least 100 days after denervation. This model system permits alimentary function and consistent weight gain among denervated animals.

The persistence of aganglionosis in our model is noteworthy. Sato and colleagues originally described chemical ablation of the enteric nervous system with BAC in 1978.²¹ Since then, BAC-denervation has been used to simulate Hirschsprung's disease in several stem cell transplantation studies.^{11,13,15} Aganglionosis in these studies was achieved by exposing BAC to the colorectum in continuity. Spontaneous regeneration of enteric ganglia has been reported under those circumstances as early as 2 months after denervation.³²⁻³⁴ Our procedure appears to prevent this spontaneous regenerative phenomenon. Furthermore, ours is the first model of colonic aganglionosis to yield consistent weight gain in denervated animals.

Our model system resembles a Thiry-Vella loop, in which each end of a bypassed, isolated segment of intestine is brought to the skin as a stoma; however, in our system the isolated segment is closed and replaced within the peritoneal cavity. While Thiry-Vella loops have proven useful for studies of pharmacology and intestinal absorption, we determined that there are two distinct advantages of maintaining an intraabdominal closed loop in our cell

therapy recipient model. First, the skin is a source of stem cells that may spontaneously migrate and engraft within denervated intestine.^{16,17} Stem cells from the stomal interface may confound the denervation process. Second, the degree of smooth muscle hypertrophy generated by our closed loop system facilitates cell injection. We anticipate that Thiry-Vella loops will be of great utility in future studies of stem cell delivery and post-transplant monitoring.

In our study, we observed a 93% overall increase in muscularis propria thickness in the experimental colon segment. Our colleagues also demonstrated a similar overall extent of smooth muscle hypertrophy in the jejunum and in the colon.³⁵⁻³⁷ Prior studies have shown varying degrees of jejunal longitudinal and circular muscle augmentation in response to BAC; however, the net effect appears to favor the longitudinal muscle layer.^{26,32,38,39} In this study, we have demonstrated dissimilar extents of hypertrophy in the circular (108%) and longitudinal (49%) smooth muscle. In the context of prior studies, we infer that the circular smooth muscle hypertrophy we observed results in large part from the isolation component of the procedure. Specifically, reflex concentric circular muscle hypertrophy and segmental dilation may develop from the mounting radial pressure of mucus accumulating within the colonic lumen. Hypertrophy in the longitudinal muscle layer may result from a combination of chemical and mechanical influences.

Our observations illustrate several limitations in this field of scientific inquiry. Primarily, BAC denervation may spare small nervous remnants. Prior studies suggest that BAC ablates over 90% of myenteric neurons upon trans-serosal exposure; however, submucosal ganglia and intramuscular axons appear relatively unaffected soon after denervation.²⁵ In this study, while nearly all myenteric ganglia were ablated, we discovered few persisting on the mesocolonic side of the colon wall. We surmise that our gauze wrap may have incompletely enveloped this small portion of the colon, and as a result these ganglia were unexposed to BAC. Our IHC analysis was insufficient to conclude that BAC exerted an ablative effect upon submucosal ganglia. This issue imposes a limitation upon future regenerative studies, as submucosal ganglion-mediated secretory function may not be attributable to cell therapy in chemically denervated recipient colon.

The potential functionality of hypertrophied colonic muscle upon enteric nervous reconstitution has yet to be investigated. In cell therapy experiments, recipient muscularis hypertrophy creates a broad recipient target, which is advantageous to the injection technique.^{14,17} Furthermore, preservation of smooth muscle contractility and absence of peristalsis has also been demonstrated after BAC denervation.²⁸ This model system is useful for both anatomical and physiological outcomes of cell therapy. Transplanted cells may be identified within intact layers of the colonic wall upon histological analysis, and current studies are underway investigating how electrical stimuli and sensors may monitor contractile physiology. Importantly, this model permits these experiments over long intervals of time. A Thiry-Vella denervation model may be better suited to studies of absorption and secretion in the experimental segment. Further studies are necessary to delineate the histological and physiological factors that may affect the outcomes of cell therapy for Hirschsprung's disease.

In conclusion, this disease model has the advantages of durable colonic aganglionosis and preserved animal alimentary function. With this new model system, long-term studies of cell-based therapy for Hirschsprung's disease are now possible. Future studies should target recipient colonic motility, absorption, secretion and cell-transplant physiology.

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Figure 1. Colonic isolation and denervation procedure. A) Identification of the descending colon; B) The descending colon is transected twice, 1 cm apart; C) The transected ends of the colon are approximated, and the bypassed isolated segment is exposed to benzalkonium chloride (BAC); D) Colonic continuity is restored with a end-to-end bypass colocolostomy, and the ends of the isolated segment are closed.

Figure 2. Postoperative evaluation. A) Laparotomy image at tissue retrieval, 50 days post-denervation. The isolated denervated colonic segment is indicated with an asterisk. An: Anastomosis site, Caud: caudad direction, Ceph: cephalad direction, DC: descending colon; B) Average postoperative animal weights over time.

Figure 3.

Muscularis thickness in normal and isolated denervated colon, 50 days post-denervation. BAC: benzalkonium chloride-treated colon, CM: circular smooth muscle layer, LM: longitudinal smooth muscle layer.

*Differences in CM thickness, $P < 0.0001$

†Differences in LM thickness, $P < 0.0001$

‡Differences in overall muscularis propria thickness, $P < 0.0001$

Figure 4.

Histological evaluation of normal and isolated denervated colon, 100 days post-denervation. A) Hematoxylin and eosin (H&E) stain of normal colon; B) H&E stain of denervated colon. CM: circular smooth muscle, LM: longitudinal smooth muscle, M: mucosa, S: submucosa; C) Glial fibrillary acid protein (GFAP) expression in normal colon; D) GFAP expression in denervated colon; E) β -III-tubulin (TUJ1) expression in normal colon; F) TUJ1 expression in denervated colon. GFAP appears green, TUJ1 appears red, DAPI appears blue, and scale bars represent 100 μ m. G) Cross-section of normal colon; H) Cross-section of denervated

colon. TUJ1 appears red and scale bars represent 100 μ m. Myenteric ganglia are denoted with arrowheads. L: colonic lumen, Me: mesocolon, MP: muscularis propria, Mu: mucosa, S: submucosa.