Transplantation of Neonatal Gut Neural Crest Progenitors Reconstructs Ganglionic Function in Benzalkonium Chloride-Treated Homogenic Rat Colon

Wei Kang Pan, Ph.D., Bai Jun Zheng, Ph.D., Ya Gao, M.D., Ph.D., Hong Qin, Ph.D., and Yong Liu, Ph.D.
*Department of Pediatric Surgery, Second Affiliated Hospital, Xi’an Jiaotong University College of Medicine, Xi’an, China; †Department of Surgical Oncology, First Affiliated Hospital, Xi’an Jiaotong University College of Medicine, Xi’an, China; and ‡Institute of Neurobiology, Xi’an Jiaotong University College of Medicine, Xi’an, China

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Background. To value the possibility and the future feasibility of the use of autograft cells transplantation in disorders of the enteric neural system, we postulate that isolated neonatal nongenetically modified neural crest progenitors could survive and differentiate into neurons and glia in homogenic denervated rats and, therefore, restore partial intestinal function after transplantation.

Methods. Neural crest progenitors were isolated from neonatal rats. After passages, the cells were labeled with CM-DiI. The labeled cells were then delivered into the muscular distal denervated colon of rats whose neural plexuses were eliminated using benzalkonium chloride. The treated colons of recipients were harvested at 1, 4, and 8 wk, and identified by immunofluorescent staining. The physiologic and functional improvements on treated colons were well examined after transplantation 8 wk.

Results. Progenitors could generate neurospheres and differentiate into neurons and glia in vitro. After transplantation, red fluorescent cells were observed in the injected tissue for up to 8 wk, and they differentiated into neurons and glia in the host colon. Functional examinations indicated that symptoms and intestinal dysfunction of the denervated model were reversed.

Conclusions. We provide herein further evidence that autologous cell transplantation is a feasible therapy for enteric nervous system disorders.

Key Words: enteric neural crest progenitors; denervated colon; homogenic rat; cell transplantation; intestinal function.

INTRODUCTION

During early embryonic development, vagal somites 1-7 and the sacral posterior somite to somite 28 migrate through the fetal bowel wall. They undergo sequential lineage restriction and, finally, differentiate into enteric neurons and glia, which give rise to the entire enteric nervous system (ENS) [1]. The ENS directs most intestinal functions, such as intestinal motility, blood flow, and epithelial secretion. Therefore, any disruption during ENS development, including disordered neural crest cell migration, localization, or differentiation, will result in dysfunction of the ENS. Moreover, pre-existing ENS abnormalities, surgical injuries or postoperative complications that affect the ENS make treatment outcomes generally unsatisfactory. Fortunately, regenerative medicine has yielded much promise in the repair or replacement of malfunctioning damaged organs and tissues. Reconstruction of the ENS to correct disorders of neural stem cells is also an intriguing possibility.

A variety of stem cells or neural progenitors derived from the gut have been reported to generate neurospheres and differentiate into neurons and glial cells with characteristic ENS phenotypes in vitro [2–11]. In addition, delivery of neural progenitors into the gut has been shown to improve bowel function [4–9]. Due to the huge diversity of cell sources, various maintenance and differentiation systems, and aganglionic
models, there is a great deal of variation in the outcomes of reconstruction therapies. These outcomes can be measured in terms of cell survival, as well as migration and differentiation of candidate cell lines. In most previous studies, stem cells were isolated from fetal rodents, postnatal human bowel, and aganglionic bowel from HSCR patients. Most of the extrinsic cells were delivered into embryonic chicks [7] or aganglionic bowel explants [8, 11]. However, there is a huge gap between organotypic culture and in vivo models. Furthermore, postnatal and embryonic neural crest progenitors are not identical in terms of immunogenicity, differentiation potential, or responsiveness to gut-specific environmental factors [12]. Until now, the biological behaviors of postnatal nongenetically modified neural crest progenitors delivered to aganglionic tissues in vivo, especially in homologous animals, had not been adequately studied. Thus, the use of this therapy to improve gut function needs to be evaluated before clinical application is possible. In the current study, we hypothesized that non-genetically modified neonatal neural crest progenitors could survive and differentiate in the denervated homologous distal colon, thereby improving the gut function of the recipient.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were bred and housed in the Laboratory Animal Center, College of Medicine, Xi’an Jiaotong University. Neonatal SD (postnatal d 5) rats were sacrificed to harvest enteric neural crest progenitors (ENCP). Adult homogenic SD rats were recipients of ENCP. All animal use complied with the regulations set forth by the Animal Research Committee (ARC) at Xi’an Jiaotong University and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication no. 85-23, revised 1985).

Cell Culture, Differentiation, and Labeling

Neonatal SD rats were sacrificed by cervical dislocation and soaked in 75% ethanol for 5 min. The mucosa-free muscular layers of the colon were stripped off and rinsed in ice-cold Hanks’ balanced salt solution (HBSS). Following mechanical dissociation by repeated trituration, the tissue samples were incubated in 0.25% trypsin (GIBCO), Grand Island, NY, USA) mixed with collagenase IV (100 U/mL, GIBCO) at 37°C for 20 min. Trypsin inhibitor (100 μg/mL, Sigma, St. Louis, MO, USA) was used to terminate the digestion. After centrifugation, the cell pellet was resuspended in Dulbecco’s modified Eagle’s culture medium (DMEM/F12, 1:1) (GIBCO) supplemented with N2 (GIBCO), B27 (GIBCO), basic fibroblast growth factor (bFGF, 20 ng/mL, Invitrogen), 2-mercaptoethanol (50 μmol/L, Sigma-Aldrich, Buchs, Switzerland), 100 U/mL penicillin, and 100 μg/mL streptomycin. The samples were then incubated in 25-cm² culture flasks at a density of 5 × 10⁶ cells/mL at 37°C in an atmosphere of 5% CO₂ overnight. The following morning, floating cells were harvested and supplemented with fresh media. Neurospheres were visible in 1 wk, and passaged every 5–7 d using 0.05% trypsin and mechanical dissociation. After passage 5–6, the neurospheres were collected and plated on poly-L-lysine (Sigma-Aldrich)-coated cover slips in 6-well plates with DMEM/F12-based media containing 10% fetal bovine serum (Sigma-Aldrich). Cells were fixed in 4% formaldehyde before immunofluorescent staining [13, 14].

Passaged cells were resuspended at a density of 1 × 10⁶/mL, incubated with CM-Dil (Molecular Probes, Burlingame, CA, USA) diluted in HBSS for 5 min at 37°C, and incubated again for another 15 min at 4°C. Labeled cells were rinsed with phosphate-buffered saline (PBS) and resuspended in fresh media.

Chemical Ablation of the Colonic ENS Model and ENCP Transplantation

Female SD rats (~200 g, n = 24) were randomly divided into three groups: control group (n = 8), denervated group (n = 8), and transplantation group (n = 8). After being fasted for 8 h with free access to water, rats were anaesthetized with chloral hydrate (4 mL/kg) i.p. The distal colon was wrapped with filter paper (width = 1 cm) and treated with 0.5% benzalkonium chloride (BAC; Sigma-Aldrich) every 3 min. About 40 min, the peritoneal cavity was thoroughly rinsed with prewarmed 0.9% normal saline. The incision was closed by using a silk suture. Histologic examination was performed to confirm the absence of myenteric and submucous neurons at 1 wk.

One week after denervation, rats in the transplantation group received cells suspended in 2 μL India ink solution, which were injected into the muscular layer of their denervated colons with a capillary micropipette (Syltech, Hangzhou, China). Cells were injected on three levels on both sides of the transitional segment and in the middle of the denervated segment. Four sites were chosen at the clock-face positions of 00:00, 03:00, 06:00, and 09:00 at each level (15 μL per site; about 1 × 10⁶ cells) (Fig 1). This procedure was repeated in triplicate.

Contrast Radiological Examination and Examination of Intraluminal Pressure of the Colon

Contrast radiological examination was performed under anesthesia with chloral hydrate (4 mL/kg, i.p.) 8 wk after transplantation. Radiographs were captured 3 min after an injection of 30% barium sulfate suspension into the colon through the anus of each rat. Six rats from each group were fasted for 12 h and given an enema to rinse the colon. The evaluation of the intraluminal pressure of the colon was performed as previously described [5]. Briefly, a small rubber balloon connected to a pressure transducer (Powerlab/4 sp; AD Instruments, Sydney, Australia) was inserted through the anus of the treated rats to examine intraluminal pressure. A stimulating rubber balloon...

FIG. 1. We preformed multipoint injections to transplant ENCP. We selected three levels on both sides of the transitional segment and in the middle of the denervated segment in which to inject cells. Four sites were chosen in the following clock-face positions: 00:00, 03:00, 06:00, and 09:00 at each level (15 μL per site; about 1 × 10⁶ cells).
was inserted through a small incision and moved to the proximal colonic segment, which was 5 cm distant from the pressure recording balloon. This procedure was repeated in triplicate.

Measuring the Weight of the Rats and the Width of the Constricted Denervated Colon

The rats' weight in each group were measured per week. After evaluation of the intraluminal pressure of the colon, rats in each group were killed by cervical dislocation. When exposed, the denervated colons were cut along the longitudinal axis. The width of the narrowest segment of the colon was measured with vernier caliper three times per rat, and the average value was recorded. This procedure was repeated in triplicate.

Pharmacologic Examinations for Contraction of Colonic Smooth Muscle

Treated colons in each group were collected and rinsed in Krebs buffer. The circular muscle was isolated and suspended along the circular axis in Petri dishes filled with Krebs at 37°C with 5% CO₂. Muscle strips were 1 cm long and 5 mm wide. Muscle tensions were detected by pressure transducer (Powerlab/4sp, AD Instruments); the resting tension was 1 g. After 40 μmol/L KCl managed 2 min to check the vasoactivity and then washed, 1 × 10⁻⁷, 1 × 10⁻⁶, 1 × 10⁻⁵, 1 × 10⁻⁴, 1 × 10⁻³ mol/L acetylcholine (ACh) were added in sequence. Contraction curves were recorded and compared between the groups by measuring the contractile amplitude and the curve of the ACh-induced reactions [15]. This procedure was repeated in triplicate.

Tissue Preparation and Immunostaining

At 1, 4, and 8 wk after transplantation, denervated colons were harvested and rinsed in PBS. The tissue was fixed in 4% formaldehyde in PBS at 37°C for 30 min and permeated in cold acetone for 10 min at −20°C. This procedure was followed by incubation in 30% sucrose overnight. Specimens were then frozen in isopentane (Sigma-Aldrich) and sectioned using a cryostat (thickness = 8 μm). Before immunostaining, 1% triton X100 (Sigma-Aldrich) was applied. The sections were blocked in 10% goat serum in PBS for 30 min at room temperature, and each section was incubated with one of the following primary antibodies: 1:50 rabbit anti-p75 nerve growth factor receptor (p75, Boisynthesis, Beijing, China), 1:500 rabbit anti-protein gene-product 9.5 (PGP9.5, Sigma), 1:800 rabbit anti-glial fibrillary acidic protein (GFAP; Neomarkers, Fremont, CA, USA), 1:200 rabbit anti-peripherin (Chemicon), or 1:100 rabbit anti-glial fibrillary acidic protein (GFAP; Neomarkers, Cambridge, UK), 1:200 rabbit anti-peripherin (Chemicon), or 1:400 rabbit anti-Sox10 (Abcam, Cambridge, UK). The sections were counterstained with 4',6-diamidine-2-phenylindole dihydrochloride (DAPI, Roche, Mannheim, Germany). Photographs were taken using fluorescence microscopy (Olympus BX51; Tokyo, Japan) and laser scanning confocal microscopy (TCS-SP2; Leica, Wetzlar, Germany).

Statistics Analysis

All data in this study are presented as mean ± SD. Statistical analysis was conducted via SPSS for Windows 11.0 software (SPSS Inc., Chicago, IL). Changes in length of denervated colon and intracolonic pressure among the three groups were tested by the nonparametric Kruskal-Wallis test. A two-way ANOVA was used to assess the different pharmacologic reactions among the groups. Differences were considered significant at P < 0.05.

RESULTS

Identification of Neurospheres and Differentiation

The morphology of the control colon was macroscopically normal (Fig. 4A), and PGP9.5 immunofluorescence revealed normal myenteric and submucosal plexuses (Fig. 4C). However, we found an obviously narrowed segment in the BAC treated-colons. Constricted segments were accompanied by distended proximal colon, which was filled with massive amounts of feces (Fig. 4B). Enteric ganglia were absent in the BAC-treated bowel segments 1 wk after denervation (Fig. 4D).

Survival and Differentiation of Transplanted Cells in the Chemically Denervated Colon

The labeled cells were clearly visible 1 wk after transplantation. The triple labeling of p75, CM-DiI, and DAPI indicated that the grafted cells remained in the undifferentiated state (Fig. 5A–D). Four weeks after transplantation, the staining of the p75-positive cells indicated that the some foreign cells remained undifferentiated, (Fig. 6A–D). Eight weeks after transplantation, CM-DiI/peripherin-positive (Fig. 7A–D) and CM-DiI/GFAP-positive cells were observed in denervated colon (Fig. 8A–D). The inset showed the CM-DiI-specific cells are extrinsic (arrowed).

Improvement of Morphology and Motility in Transplanted Colon

Contrast Radiological Photograph of the Colon

Contrast radiological examination revealed a narrowed segment accompanied by distended proximal colon filled with massive amounts of feces in the denervated group, and barium sulfate was pushed with difficulty into the treated colon (Fig. 9B) compared with the control group (Fig. 9A). The symptoms of six rats were
relieved in the transplantation group, and the affected colons had improved morphology 8 wk after transplantation (Fig. 9C).

Changes in Intraluminal Pressure of the Colon

Upon stimulation, some control-group colons reflexively contracted and intracolonic pressure increased. In contrast, the denervated group did not reflexively respond to inflation. Also, the transplantation group colons behaved similarly to the control group. Statistical analysis demonstrated the intracolonic pressure in the denervated group was significantly lower than both the control (0.081 ± 0.091 versus 2.152 ± 0.185, P < 0.01) and transplantation groups (1.380 ± 0.276, P < 0.01). There was no significant difference between

FIG. 2. Characterization of neonatal ENCP. After five to six passages, ENCP were harvested from neonatal rat gut tissue. Neurospheres contained numerous cells immunopositive for the neural crest cell and neural progenitor markers nestin (A) and p75 (B). Cells were also immunopositive for PGP9.5 (C) and Sox10 (D) staining. The nuclei were counterstained with PI (red). Scale bar: 100 μm.

FIG. 3. Identification and differentiation of neonatal ENCP in vitro. Differentiated neurons and glial cells were identified by TuJ1 (A), GFAP (B), and PGP9.5 (C). The nuclei were counterstained with DAPI (blue). Before transplantation, the neurospheres and single cells were labeled with CM-DiI (D). Scale bar: 100 μm.
the control and transplantation groups ($P > 0.05$; Fig. 10).

**Measurement of the Weight of the Rat and the Width of the Constricted Denervated Colon**

After 1 wk treated by BAC, the aganglionic rats began to be less active, depressed, and anorexic. The weight of the treated rats showed various degrees of loss. But the cell transplantation could relieve symptoms and reverse the weight loss (Fig. 11A). After 8 wk, the weight of the denervated group was significantly lower than both the control ($134.8 \pm 3.8$ versus $222.1 \pm 15.3; P < 0.01$) and transplantation groups ($versus 181.7 \pm 6.9; P < 0.01$). There was no significant difference between the control and transplantation group ($P > 0.01$). The width of the...
constricted colon in the denervated group was significantly shorter compared with either the control (0.858 ± 0.085 versus 1.19 ± 0.897; P < 0.01) or transplantation group (versus 1.082 ± 0.103; P > 0.05). There was no significant difference between the latter two groups (P < 0.01; Fig. 11B).

Colonic Smooth Muscle Contraction Induced by Acetylcholine

In the control group, the contraction of colonic smooth muscle increased as the concentration of acetylcholine increased. In contrast, the denervated colon maximally contracted when exposed to low concentrations of...
acetylcholine (1 μmol/L). The response of colonic smooth muscle to acetylcholine was restored in the transplantation group. Dose-effect curves showed that the response in the denervated group was more significantly sensitive than either the transplantation group ($P < 0.01$) or control group ($P < 0.01$). However, the transplantation group was similar to the control group ($P > 0.05$; Fig. 12).

**FIG. 8.** Representative photographs from the rats 8 wk (A–D) after transplantation, demonstrating that detected glial cells were derived from neonatal ENCP. Triple labeling of rat GFAP-specific antibodies [(A), green], CM-DiI [(B), red], and DAPI [(C), blue] showed a number of glial cells (arrowed) derived from neonatal ENCP 8 wk after transplantation. The inset showed the CM-DiI-specific cells are extrinsic. Scale bar: 100 μm.

**FIG. 9.** Radiographs were taken 8 wk post-transplantation. Contrast radiologic examinations of the control group (A), denervated group (B), and transplantation group (C) are shown. The arrows indicate the BAC-treated segments.
DISCUSSION

In this study, we demonstrate that extrinsic, non-genetically modified neonatal ENCP could survive and differentiate in homogenic denervated colon. Furthermore, this cellular therapy could relieve the symptoms and improve the function of the denervated model. In previous studies, a range of stem cells could differentiate into neurons, such as embryonic stem cells [16, 17], neural stem cells including central neural system-derived neural stem cells (CNS-NSC) [8], peripheral neural stem cells and enteric neural stem cells [18], as well as stem cells from other sources (mesenchymal stem cells). However, the direct use of embryonic stem cells is ethically restricted, and it is also possible to produce negative complications, e.g., teratoma-like growths, which have been reported previously by others [19]. Moreover, most adult stem cell lineages (such as bone marrow, skin, and adipose tissue) may require additional genetic reprogramming to produce an enteric neuronal phenotype in vitro and in vivo, the mechanisms of which are poorly understood [4]. Obviously, enteric neural progenitors may hold stronger potentials than CNS-NSC to differentiate into enteric neuron or glia because of their phenotypic similarity. In addition, using postnatal ENCP not only helps avoid ethical issues, but also the ultimate goal is to obtain autologous cells from patient with enteric neurological diseases who are undergoing gut surgery or routine endoscopic examination. Evidence that autologous cell-based therapies are a feasible treatment is supported by a recent study in which adult human enteric neural progenitors were expanded and differentiated in vitro [11]. However, it should be noted that whether these cells which were cultured from the patients have gene defect or not. The aganglionic animal model first described by Sato [20, 21], which was established using BAC in 1978, was widely used for neurological research. Our data indicated that labeled neonatal ENCP could survive and differentiate into neurons and glial cells in the denervated rat colon up to 8 wk post-transplantation. Previous study has indicated that most of stem cells remain undifferentiated [5] after transplantation 2 wk. In our study, we found that the staining of p75-positive cells was still observed after transplantation 4 wk. Obviously, there is a notable difference between embryo progenitor and neonatal progenitor.

Previous studies have demonstrated that BrdU staining can be used to track stem cell engraftment

FIG. 10. Histogram shows the changes in intraluminal pressure of the three groups. Secondary to stimulation, some colons in the control group reflexively contracted, and the intracolic pressures increased. In contrast, the denervated group did not respond to stimulation by inflation, but the transplantation group was similar to the control group. The intracolic pressure of the denervated group was significantly lower than both the control and transplantation groups. *P < 0.01 control group versus transplantation group as determined by the Kruskal-Wallis test (n = 6 each group).

FIG. 11. (A) The change curve of weight in three groups. After 1 wk treated by BAC, the weight of the treated rats in transplantation group and denervated group showed various degrees of loss; but the cells transplantation could reverse the weight loss. Data are presented as mean ± SD. After 8 wk, the weight of the denervated group was significantly lower than both the control and transplantation groups. *P < 0.01 versus control group and transplantation group by one-way ANOVA (n = 6 each group). (B) The width of the narrowest (relatively in control group) colonic segment among the three groups; * P < 0.01 versus control group and transplantation group by non-parametric Kruskal-Wallis test (n = 6 each group).
However, BrdU can yield false positive results due to the incorporation of BrdU from lysed dead cells or otherwise directly from the medium [2]. To prove that the injected cells were of extrinsic origin, we used CM-DiI, a red-fluorescent membrane marker, to track these cells. Our data showed that although the intensity of fluorescence gradually decreased, we could still find the labeled cells 8 wk after transplantation. Unfortunately, we did not detect the labeled cells after 9 wk. Possible reason is that the red-fluorescent dye decays to a undetectable level over time in our animal model, so it is critical to find out the best cell labeling in order to facilitate the future study on long term cells transplantation.

To evaluate the recovery of function after transplantation, we performed barium enema radiography, examination of the intraluminal pressure of the colon, and evaluation of pharmacologic responsiveness of colonic smooth muscle contraction; all of which are commonly used clinical techniques. A contrast radiological examination and measuring the weight of the rats and the width of the constricted denervated colon indicated that the ENS regained regulated colonic function, and morphological observation showed that symptoms were in the denervated colon were also relieved. Examination of the intraluminal pressure of the colon showed that extrinsic cells had reconstructed the ENS via either a direct effect or an indirectly mediated effect of endogenous factors that influenced smooth muscle contraction. The foreign-cell therapy had induced decreases in the intraluminal pressure in the denervated colon. ACh combined with muscarinic receptors distributed on the gastrointestinal smooth muscle cell membranes caused the contraction of gastrointestinal smooth muscle and showed a dose-effect relationship [23]. Pharmacologic examinations revealed that there was increased sensitivity to acetylcholine in the colonic smooth muscle of the denervation group. Also, spontaneous contraction induced by low concentrations of acetylcholine in the denervated group could reach maximum contractile tension. BAC strengthened spontaneous contraction of circular muscle of colon. One explanation is that this is secondary to automatic regulation achieved by either decreasing acetylcholinesterase activity or increasing the quantity or affinity of the acetylcholinergic receptor [20, 21]. Most importantly, these changes were recovered after neonatal ENCP transplantation which could differentiate inhibitory neurons and restrict the disorder of spontaneous contraction. In a previous study, it was reported that transplanted colonic smooth muscle displayed changes in response to electrical field stimulation in comparison to denervated colon [5], a finding consistent with our study.

Until now, the migration capability of foreign progenitors and the migration distance of the aganglionic colon in the host colon remain unknown. Previous studies suggest that longitudinal migration of transplanted cells within recipient embryonic gut is limited to a few millimeters at best [11]. Moreover, it is well-known that the capability of proliferation and differentiation in postnatal progenitors is less than embryonic progenitors [12]; there is a significant difference in each cell type’s responsiveness to the gut-specific niche. So we attempt to perform multipoint injection. Still, the functional improvement of the denervated colon proven by our method was reasonable. We speculated that a multipoint injection may be favorable to leverage intercellular communication and the endocrine activity.

In the preliminary experiments, we used sutures to label the injection sites. However, when the animals were sacrificed just after transplantation, we could not clearly identify the injection site. To detect the injection sites weeks after in vivo injection, and to avoid rejection caused by labeled foreign materials, we used India ink to mark the injection sites (communication with Qin [24]). It turned out that labeling with India ink was convenient and precise.

In conclusion, our data provided further proof that neonatal enteric neural crest progenitors can survive, differentiate, and even partially restore function in homogenic aganglionic recipient colon. This result is necessary experimental evidence supporting the use of autologous cell-replacement therapy in ENS disorders. However, additional work will be required to investigate whether or not the recipient’s gut can maintain a beneficial microenvironment for long periods of time, and it is also important to evaluate the migration capability of grafted cells.
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