Journal of Pediatric Surgery 51 (2016) 1581–1584

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Original Articles

Enteric nervous system stem cells associated with thickened extrinsic fibers in short segment aganglionic Hirschsprung's disease gut are absent in the total colonic and intestinal variants of disease☆,☆☆,★

George Bethell [a,b], David Wilkinson [a,b], David Fawkner-Corbett [a,b], Angelica Mesa [a], Rajeev Shukla [c], David Edgar [a], Simon Kenny [a,b,*]

[a] University of Liverpool Institute of Translational Medicine, Liverpool, United Kingdom
[b] Department of Paediatric Surgery, Alder Hey Children's Hospital NHS Foundation Trust, Liverpool, United Kingdom
[c] Department of Pathology, Alder Hey Children's Hospital NHS Foundation Trust, Liverpool, United Kingdom

ARTICLE INFO

Article history:
Received 10 January 2016
Received in revised form 1 June 2016
Accepted 4 June 2016

Key words:
Hirschsprung's disease
Stem cell therapy
Enteric nervous system
Neural crest cells

ABSTRACT

Background/purpose: Despite current treatments patients with Hirschsprung's disease (HSCR) suffer significant long-term morbidity. Therefore, there is increasing interest in adjunctive therapies, such as using enteric nervous system stem cells (ENSSC), isolated from typical aganglionic bowel. The source of these cells is unclear however it is hypothesized that they are present in the thickened nerve trunks in aganglionic short and long segment HSCR gut. These cells should therefore be absent in total colonic and pan intestinal HSCR where these thickened fibers are absent.

Methods: Cells were isolated from samples of short segment HSCR gut (n = 18) and total colonic and total intestinal HSCR gut (n = 2). Acetylcholinesterase histochemistry confirmed the presence/absence of thickened nerve trunks. P75 immunofluorescence highlighted ENSSC at isolation and after 10 days in culture in both groups.

Results: ENSSC were not isolated or cultured from total colonic and total intestinal HSCR gut where thickened nerve trunks were absent. In contrast 10.0% (+/−1.9 SEM) of cells from short segment HSCR gut were ENSSC at isolation rising to 22.7% (+/−2.9 SEM) after 10 days in culture.

Conclusions: These results associate ENSCC with thickened nerve trunks and also suggest that the aganglionic bowel segment in total colonic and intestinal HSCR cannot be used as a source of ENSCC for adjunctive therapy.

© 2016 Elsevier Inc. All rights reserved.

Hirschsprung's disease (HSCR) is a congenital disorder in which the enteric nervous system (ENS) fails to develop in the distal bowel. The tonic contraction of this aganglionic gut and dilation of the proximal ganglionic section results in a functional obstruction, commonly presenting as acute life-threatening obstruction in the neonatal period. The length of affected bowel is variable: short segment HSCR is typically limited to the rectum/sigmoid and accounts for more than 80% of cases [1]. Long segment HSCR extends proximally beyond the rectosigmoid junction and has an incidence of 15–20%. In total colonic disease the whole of the large intestine is affected which accounts for 5% of HSCR. Total intestinal disease extends to at least the duodenum and is found in around 1 in 500,000 live births [2,3].

Recently our group published observations showing that enteric nervous system stem cells (ENSSC) can be isolated and cultured from the aganglionic segment of gut in short and long segment HSCR [4]. Furthermore, the data indicated that the source of these ENSCC was the thickened nerve trunks characteristic of the aganglionic regions of long and short segment HSCR. Implantation of aganglionic ENSSC into ex vivo mouse gut behaved similarly to ganglionic ENSSC and demonstrated cell migration and differentiation, restoring normal patterns of contractility. These findings highlight a potential therapeutic use for the HSCR patient's own ENSSC to address long-term continence issues experienced following pull-through surgery, either as an adjuvant injection into the internal anal sphincter, or by stimulation of these quiescent ENSSC within aganglionic gut to allow normal function [5,6]. In order to control and limit the proliferation of ENSSC post implantation and also understand how to stimulate ENSSC in vivo it is of paramount importance to fully characterize the cellular source in aganglionic gut.

It has been shown that thickened extrinsic nerve trunks are present in aganglionic short and long segment HSCR gut but are absent from aganglionic total colonic and total intestinal HSCR bowel [7,8]. We

☆ Author contributions: Study design and conception (GB, DW, DFC, DE, SK); data collection, analysis and interpretation (GB, DW, DFC, AM, RS, DE, SK); first draft of manuscript (GB); subsequent drafting of manuscript (GB, DW, DFC, DE, SK); approval of final manuscript (GB, DW, DFC, AM, RS, DE, SK).

☆☆ No payment was received for drafting manuscript.

★ Conflict of interest: No conflicts of interests for all authors identified above.

* Corresponding author at: University of Liverpool Institute of Translational Medicine, Child Health, Prescot Street, Liverpool, L69 3BX, United Kingdom. Tel.: +44 (0)151 794 5543.

E-mail address: kennyse@liverpool.ac.uk (S. Kenny).

http://dx.doi.org/10.1016/j.jpedsurg.2016.06.006
0022-3468/© 2016 Elsevier Inc. All rights reserved.

hypothesized that if the aganglionic ENSSC are indeed derived from thickened nerve trunks it will not be possible to isolate or culture ENSSC from aganglionic gut in total colonic or total intestinal HSCR disease where thickened nerve trunks are absent. The demonstration here that the ENSCC cannot be isolated from total colonic or total intestinal HSCR more closely defines the potential use of aganglionic HSCR gut as a source of cells for autologous treatment of the disease [4].

1. Methods

1.1. Human tissue

Ethical approval for the isolation of human enteric nervous system stem cells was given by the UK North West 3 Research Ethics Committee (Ref: 10/H1002/77). Written parental consent was obtained before samples were taken. Human aganglionic gut specimens were obtained from 18 patients with short-segment Hirschsprung's disease, 1 with the total colonic disease and 1 with total intestinal disease. Around $1cm^2$ of aganglionic gut was obtained from each HSCR patient at the time of stoma formation or pull-through procedure. The presence or absence of thickened nerve trunks and aganglionosis were confirmed with acetylcholinesterase (AChE) and calretinin histochemistry on frozen specimens, respectively, by the hospital pathology department. Samples were transferred to the lab in saline soaked sterile gauze on ice.

1.2. Isolation of cells

The sample was removed from the saline gauze and washed twice in 10 ml phosphate buffered saline (PBS) under sterile conditions in a 90 mm petri dish (Sterilin, Newport, UK). A final wash of PBS supplemented with Gentamycin at a concentration of 50 μg/ml took place. A dissecting microscope (M165FC, Leica Microsystems, Bucks, UK) was then used to separate the mucosa and sub mucosa from the specimen along with removal of any blood vessels and any adipose tissue from the serosal surface. The remaining tissue, which is the muscular layers, was then manually chopped into $1mm^2$ pieces and transferred to a 15 ml centrifuge tube (E1415–0200, Starlab, Hertfordshire, UK). The sample was centrifuged at 150 g for 5 min in order to remove the PBS/Gentamycin solution. 2 ml of Dispase (Gibco®) and 2 ml of Collagenase IV (Gibco®), both at a concentration of 1% (w/v), were added to the sample and left in a water bath for 1 h at 37 °C. Every 15 min the sample was gently triturated using a 5 ml glass pipette. After 1 h the sample was centrifuged at 150*g* for 5 min and fresh enzymes were added. This cycle would be repeated 2–3 times depending on the appearance of the sample when 10 μl was taken and viewed under an inverted microscope (Nikon TMS-F, Nikon UK, Surrey, UK). Once there are no visible pieces remaining, centrifugation produced a pellet of cells which was re-suspended in PBS and passed through a 100 μm cell strainer (Falcon®, BD Biosciences, UK). Following centrifugation a final pellet was re-suspended in Dulbecco's modified Eagle medium (high glucose, 4.5% w/v), supplemented with 199 media (20% v/v), heat-inactivated horse serum (7% v/v), 100 units/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies, Paisley, UK). 10 μl of the sample was then taken and added to 10 μl 0.4% (w/v) Trypan blue, before counting cells using a hemocytometer. Between 2 and 4×10^7 cells were then plated on 60 mm adherent dishes (Nalge Nunc, NY, USA) with 6 ml of the above medium supplemented with 0.5 μg/ml amphotericin B (Fungizone®, Gibco®) and 50 μg/ml Gentamicin (Gibco®). The cultures were kept in an incubator at 37 °C with 5% CO_2 added to air for 10 days.

1.3. Immunofluorescence of cells in culture using chamber well slides

At isolation, before a cell suspension has been cultured, the volume of the suspension to contain 20,000 cells was calculated. Alternatively, cells in culture had their supernatant removed by centrifugation at 150*g* for 5 min in a 15 ml centrifuge tube. The pellet produced was then re-suspended in 1 ml of 0.05% (w/v) Trypsin (Sigma-Aldrich) and added to the culture dish along with a further 2 ml of Trypsin. After 10 min at 37 °C in an incubator, gentle manual dissociation occurred using a 1 ml pipette. The suspension was then centrifuged to produce a pellet which was then re-suspended in 1 ml of media. A cell count took place and the volume needed to give 20,000 cells was calculated. The remaining cells were re-cultured on a fresh 60 mm adherent dish. 200 μl of PBS containing 5% (v/v) Fibronectin (Merck Millipore) was added to each chamber of the adherent chamber well slide (Thermo Scientific). This was then incubated at 37 °C for 1 h before the PBS containing Fibronectin was removed and a brief PBS wash took place. 20,000 cells were then re-suspended in 500 μl of the same medium as they were in when in culture and added to each well. The cells were then incubated overnight at 37 °C with 5% CO_2. The following day a PBS wash took place and 200 μl of 4% (w/v) paraformaldehyde was added to each well to fix the cells. Following a PBS wash, 200 μl of immunofluorescence blocking buffer (IBB) (PBS ($CaCl_2$/$MgCl_2$ free, Gibco®) was supplemented with 20% v/v Goat serum (Gibco®), 0.1% w/v NaN3 (Sigma-Aldrich) and 1% w/v bovine serum albumin (Sigma-Aldrich)) was added to each well and left for 1 h. The primary antibody P75 (ab3125, Abcam) was added to each well at a concentration of 1:1000 diluted in IBB. At least one well for a control received IBB alone. The slide was then left overnight refrigerated at 2–4 °C. A PBS wash took place and Alexa Fluor 488 (Life Technologies) antibodies diluted to a concentration of 1:1000 were added. The slide was then left at room temperature in the dark for 1 h. A final PBS wash took place and the chamber was separated from the glass slide using the tool supplied with the chamber well slides. The slide was left until dry and a drop of ProLong® Gold antifade reagent with DAPI (Life Technologies) was placed over each well on the slide before covering with a cover slip (Thermo Scientific). The slide was then left at room temperature in the dark overnight to dry before viewing and imaging. This method is as previously described [9,10].

Slides were viewed using a Leica DM IRB Inverted microscope (Leica Microsystems). Images were captured using Leica Application Suite V4.3 (Leica Microsystems). Manual random sampling of rows of cells occurred using the 20× objective and the number of fluorescing cells and nuclei identified by DAPI were counted. Each row contained between 100 and 300 cells and the mean of the three rows was taken to give a percentage of cells expressing the antigen in the sample. Adobe Photoshop CS3 (Adobe Inc., CA, USA) was used for merging images.

1.4. Statistical analysis

GraphPad Prism version 5 (GraphPad Software Corporation, CA, USA) was used to perform statistical analyses. Mann–Whitney U was used for continuous non-parametric data. Standard error of the mean was used for comparing outcomes of repeated experiments.

2. Results

Thickened nerve trunks were present in the aganglionic short segment HSCR gut ($n = 18$) (Fig. 1 A + C) and absent from the total colonic and total intestinal gut samples as demonstrated by acetylcholinesterase staining ($n = 2$) (Fig. 1 B + D). Immunohistochemical staining with calretinin shows the absence of ENS ganglia in both extents of disease (Fig. 1 A + B).

After overnight incubation no cells from either the total colonic or total intestinal HSCR gut expressed P75, showing an absence of ENSSC (Fig. 2 E + F). In contrast, 10.0% (+/−1.9 SEM) of the cells from short segment gut did express P75 (Fig. 2 A + B). After 10 days in culture no cells from either total colonic or total intestinal gut expressed P75 (Fig. 2 G + H) whereas 22.7% (+/−2.9 SEM) of the cells in the sample from short segment gut expressed the ENSSC marker (Fig. 2 C + D). These levels of expression in short segment HSCR are consistent with previous work [4].

Download English Version:

https://daneshyari.com/en/article/4154661

Download Persian Version:

https://daneshyari.com/article/4154661

Daneshyari.com