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ORIGINAL ARTICLE

Altered expression of caveolin-1 in the colon of patients with Hirschsprung's disease

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Abstract

Background/purpose The pathogenesis of Hirschsprung's disease-associated enterocolitis (HAEC) is unclear. Caveolin-1 (Cav-1) regulates the functions of different nitric oxide synthase (NOS) isoforms, which play critical roles in inflammation and intestinal epithelial barrier function. We designed this study to investigate the hypothesis that Cav-1 expression is altered in the bowel of patients with Hirschsprung's disease (HSCR).

Methods HSCR tissue specimens ($n=10$) were collected at the time of pull-through surgery and control samples were obtained at the time of colostomy closure in patients with imperforate anus ($n=10$). qRT-PCR analysis was undertaken to quantify Cav-1 gene expression, and Western blot analysis was undertaken to determine Cav-1 protein quantification. Immunolabelling of Cav-1 proteins was visualized using confocal microscopy.

Results qRT-PCR and Western blot analysis revealed that Cav-1 was significantly downregulated in the aganglionic and ganglionic colon of patients with HSCR compared to controls ($p<0.01$). Confocal microscopy revealed a markedly decreased expression of Cav-1 in colonic epithelium of aganglionic and ganglionic bowel of patients with HSCR compared to controls.

Conclusion To our knowledge, this is the first report of significantly decreased Cav-1 expression in patients with HSCR. Decreased expression of Cav-1 in the bowel of HSCR may increase susceptibility to HAEC in HSCR.

Keywords Hirschsprung's disease · Enterocolitis · Caveolin-1

Introduction

Hirschprung's disease-associated enterocolitis (HAEC) is a serious complication of Hirschsprung's disease (HSCR) with a reported incidence of 20–58% [1, 2]. HAEC can occur before or after a properly performed pull-through operation. HAEC occurring following a pull-through operation has been reported in 5–44% of HSCR patients [3]. The pathogenesis of HAEC is still not fully understood.

Nitric oxide (NO) mediates intestinal homeostasis and is an important inflammatory mediator and plays a critical role in intestinal barrier failure observed in many inflammatory diseases of the intestine [4]. NO is produced from arginine in a reaction catalyzed in the intestine by NO synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). NO is believed to modulate water and electrolyte transport, as well as mucosal permeability [5]. Low levels of NO regulate vascular tone and mucosal blood flow in a cyclic GMP- and neuron-dependent manner [5]. The beneficial effects of constitutively low production of NO include the maintenance of mucosal capillaries, mucosal homeostasis, protection from oxidative stress and promotion of leukocyte adhesion to the endothelium [5–9]. High levels of NO seen during inflammation exert detrimental effects on intestinal barrier function leading to increased bacterial translocation, impaired mitochondrial function and epithelial restitution, and decreased leukocyte recruitment by the endothelium [5, 10–16].

Recently, a novel protein, caveolin-1 (Cav-1) has been described, which regulates the functions of various NOS isoforms (eNOS, nNOS and iNOS), and intestinal growth factors (IGF-1, IGF-2 and EGF) [17]. Both NOS isoforms and intestinal growth factors play critical roles in inflammation and intestinal epithelial barrier function, respectively

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[17]. It is known that decreased Cav-1 expression regulates iNOS activity, thus leading to enhanced reactive oxygen species (ROS) production, and this increased ROS production induces endothelial dysfunction in the swine model [18]. It has recently been reported that Cav-1 knockout mice exhibit an up-regulated inflammatory response in a murine chemical-induced colitis model, which suggests that endothelial Cav-1 mediates angiogenesis in experimental colitis [19]. We designed this study to investigate the hypothesis that Cav-1 expression is altered in the bowel of patients with HSCR.

Materials and methods

Tissue collection

Ethical approval for collection of specimens was in place from both the institutions participating in the study (Our Lady's Children's Hospital Ethics Committee, GEN292.12; Temple Street Children's University Hospital Research and Ethics Committee, 13.003). Full-length resected bowel specimens obtained during pull-through operation for HSCR were collected from ten patients. Four of these patients had a history of preoperative HAEC. Resected tissue included aganglionic and ganglionic segments. Ganglionic specimens were taken from the most proximal margin of the resected pull-through specimen while aganglionic samples were taken from the most distal margin of the resected specimen. Healthy control colonic specimens were obtained from the proximal colostomy limb at the time of stoma closure in patients with imperforate anus ($n = 10$). Tissue specimens were stored in three ways following collection. One segment of each specimen was fixed in formalin at room temperature, for paraffin embedding and immunochemistry. A second segment was snap frozen in a mold containing optimal cutting temperature (OCT) medium and stored at − 80 °C for immunofluorescence and confocal microscopy. The remaining segment was stored at − 80 °C for RNA extraction and protein extraction.

RNA isolation

TRIzol reagent (Invitrogen) was used for the acid guanidinium thiocyanate–phenol–chloroform extraction method to isolate total RNA from HSCR and control tissues ($n = 10$ for each group) according to the manufacturer's protocol. Spectrophotometrical quantification of total RNA was performed using a NanoDrop ND-1000 UV–Vis spectrophotometer (Thermo Scientific Fisher, Wilmington, USA). The RNA solution was stored at − 20 °C until further use.

cDNA synthesis and quantitative polymerase chain reaction

Reverse transcription of total RNA was carried out at 85 °C for 3 min (denaturation), at 44 °C for 60 min (annealing) and at 92 °C for 10 min (reverse transcriptase inactivation) using a Transcriptor High-Fidelity cDNA Synthesis Kit (Roche Diagnostics, West Sussex, UK) according to the manufacturer's instruction. The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR) using a LightCycler 480 SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) in a total reaction mix of 20 µl per well. The following gene-specific primer pairs were used: Human Cav-1 (Eurofins) sense primer 5′ CGC CAT TCT CTC TTT CCT GC and Human Cav-1 (Eurofins) antisense primer 5′ CGG TGT GGA CGT AGA TGG AA. For normalization purposes, real-time RT-PCR was performed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH sense prime 5′ACA TCG CTG AGA CAC CAT GG and GAPDH antisense primer 5′ GAC GGT GCC ATG GAA TTT GC were used. After 5 min of initial denaturation at 95 °C, 55 cycles of amplification for each primer was carried out. Each cycle included denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 10 s. Relative mRNA levels of gene expression were determined using a LightCycler 480 System (Roche Diagnostics) and the relative changes in gene expression level of interest were normalized against the level of GAPDH gene expression in each sample (DDCT method). Experiments were carried out in duplicate for each sample and primer.

Protein extraction and Western blot

Specimens of HSCR colon and control colon were homogenized in RIPA buffer (Radio-Immunoprecipitation Assay, Sigma-Aldrich Ltd., Wicklow, Ireland). Protein concentrations were determined using a Bradford assay (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). A total volume of 40 µl Laemmli Sample Buffer (Sigma-Aldrich, Ireland Ltd., Wicklow, Ireland) containing 10 µg protein was loaded in the 10% SDS-PAGE gel (NuPAGE Novex Bis–Tris gels, Invitrogen, Carlsbad, USA) for electrophoretic separation. The electrophoresis was performed in MES SDS (2-(*N*-morpholino) ethane sulfonic acid, sodium dodecyl sulfate) running buffer (Invitrogen, Carlsbad, USA). Proteins were then transferred to 0.45-µm nitrocellulose membrane (Millipore Corporation, Billerica, USA) by Western blotting. Following Western blotting, the membranes were blocked with 3% bovine serum albumin [BSA, Sigma-Aldrich, Ireland (A2153-50G)] for 60 min before antibody detection. The primary antibodies, rabbit anti-Cav-1 (Abcam, Cambridge, UK, ab2910) dilution 1:1000, were used and incubation was performed overnight at 4 °C. Following extensive washing [four times in PBS (phosphate-buffered saline)–0.05% Tween] the membranes were incubated with goat anti-rabbit IgG HRP-linked secondary antibody (dilution 1:10,000, Abcam, Cambridge, UK) followed by washing (four times in PBS-0.05% Tween). Detection was performed with the ECL Plus Chemiluminescence Kit (Thermo, Fisher Scientific, Dublin, Ireland). We used GAPDH (mouse anti-GAPDH, dilution 1:1000, Abcam, Cambridge, UK) as an additional loading control. Quantification of Western blot has been conducted with ImageJ software (US National Institutes of Health, Bethesda, Maryland, USA) by measuring the density of every single band and normalizing it against the density of the corresponding GAPDH band.

Immunofluorescence

Colonic sections were embedded in OCT compound [VWR, Ireland (361603E)] and snap frozen in liquid nitrogen. Twenty-micron sections were cut and fixed in 10% neutral buffered formalin (Sigma-Aldrich, Ireland [HT501128-4L]). Cell membranes were permeabilized by rinsing in 1% w/v PBS with 1% Triton X-100. Sections were blocked in 10% BSA diluted in 1% w/v PBS with 0.05% PBST for 90 min at room temperature to prevent non-specific antibody binding. Samples were incubated simultaneously in both the primary antibodies: rabbit anti-Cav-1 (1:500, 5% BSA) (Abcam, Cambridge, UK, ab2910) and mouse anti-EpCAM (1:500, 5% BSA) (Santa Cruz, Heidelberg, Germany, sc-25308) at 4 °C overnight. Following incubation in primary antibody solution, samples were rinsed intensively in 0.05% PBST, following which they were incubated in a solution containing corresponding secondary antibodies (anti-rabbit Alexa Fluor488 (ab150073, GR226381), dilution 1:1000 and antimouse Alexa Fluor 584 (ab150116, GR232081), dilution 1:1000), (Abcam, Cambridge, UK) for 90 min at room temperature. After intensive rinsing in 0.05% PBST, samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain [Thermo Scientific, Ireland (EN62248)]. Sections were mounted with glass coverslips using Mowiol® 488 fluorescence mounting medium [Sigma-Aldrich, Ireland (81381-50G)], which was constituted according to the manufacturer's specifications. Specimens were visualized using laser scanning confocal microscopy (LSM700 Confocal Microscope, Carl Zeiss MicroImaging GmbH, Jena, Germany). Resulting images were processed using ImageJ—an open-access software available from http://imagej.nih.gov/ij/.

Statistical analyses

A one-way ANOVA and the Student's t test with Bonferroni correction were conducted to determine a statistically significant difference between aganglionic, ganglionic and healthy controls. A p value < 0.05 was considered to be statistically significant.

Data are presented as mean ± standard error. Specimens were classified into three groups: aganglionic ($n = 10$), ganglionic ($n = 10$) and healthy controls ($n = 10$).

Results

Relative mRNA expression levels of Cav-1

The relative mRNA expression levels of Cav-1 were significantly decreased in aganglionic and ganglionic (HSCR) specimens compared to normal controls ($p < 0.01$, Fig. 1).

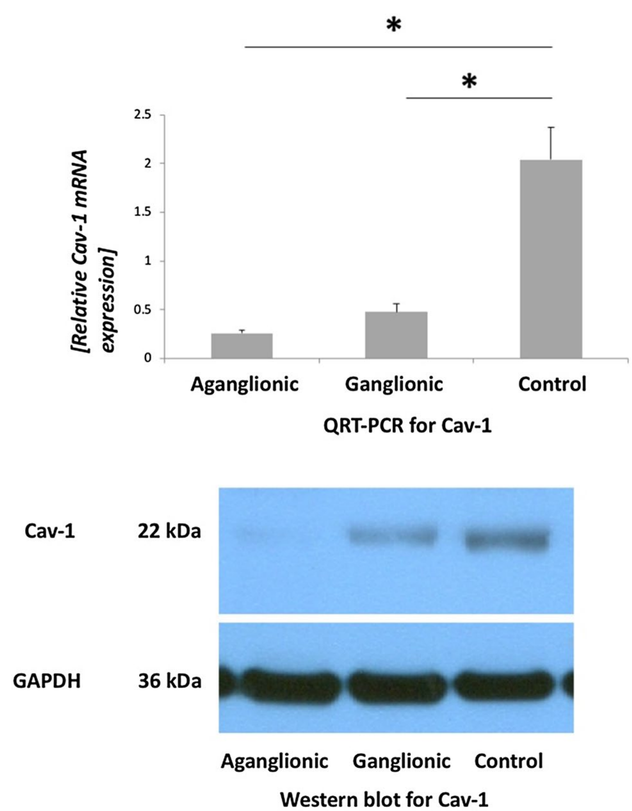

Fig. 1 qRT-PCR revealed significantly decreased relative mRNA expression levels of Cav-1 in the aganglionic and ganglionic HSCR specimens ($n = 10$) compared to normal control tissue ($n = 10$). Results are presented as mean ± SEM (*$p < 0.01$ by one-way ANOVA). Western blotting revealed significantly decreased protein expression levels of SPDEF, KLF4 and TFF3 in the aganglionic and ganglionic HSCR specimens ($n = 10$) compared to normal control tissue ($n = 10$). Equal loading of electrophoresis gels was confirmed by GAPDH staining ($p < 0.01$ by one-way ANOVA)

Western blot

Our Western blot results from three independent experiments showed that Cav-1 protein was expressed in the colon of patients with HSCR and the expression was significantly decreased in the aganglionic and ganglionic bowel compared to healthy controls ($p < 0.01$, Fig. 1). Equal loading of electrophoresis gels was confirmed by GAPDH (glyceraldehyde 3-phosphate dehydrogenase) staining of the stripped membranes.

Immunofluorescence staining and confocal microscopy

Cav-1 could be detected in the colonic epithelium of the aganglionic and ganglionic HSCR specimens and control samples. Compared with the control group, there was a significantly decreased expression of Cav-1 in the colonic epithelium of patients with HSCR (Fig. 2).

Discussion

To our knowledge, this is the first report describing significantly decreased expression of Cav-1 in the colon of patients with HSCR. In the present study, qRT-PCR and Western blot analysis revealed that Cav-1 was significantly downregulated in the aganglionic and ganglionic colon of patients with HSCR compared to controls. Confocal microscopy revealed a markedly decreased expression of Cav-1 in colonic epithelium of patients with HSCR as compared to controls. Strikingly, the altered expression of Cav-1 was not only confined to the aganglionic colon but also affected ganglionic colon which may explain why many patients continue to have episodes of HAEC and dysmotility problems despite a properly performed pull-through operation.

Caveolae, Latin for 'small caves', are microscopic invaginations of the plasma membrane known to regulate key aspects of endocytosis, transcytosis, mechano-transduction, and clustering of signaling molecules [20–24]. Caveolins have certain structural components for their formation: Cav-1, caveolin-2, and caveolin-3. Each of these proteins has specific roles which can vary from cell type to cell type [19, 25]. Cav-1 is associated with a number of biological roles in some disease conditions including sepsis, tumor, pulmonary fibrosis, lung injury and colitis [19]. Recently, Weiss et al. [19] reported that Cav-1 may play a protective role in the development of TNBS-induced colitis.

This protective effect could be due to the involvement of Cav-1 in cellular signaling and an inhibitory effect on cytokine production through various mechanisms [19, 26, 27]. During caveolae-dependent signaling, Cav-1 works as a scaffold protein which organizes various signaling complexes involved in diverse cell activities [19, 26]. As a result, a deficiency of Cav-1 may affect a number of disease processes. Cav-1 has been shown to have several

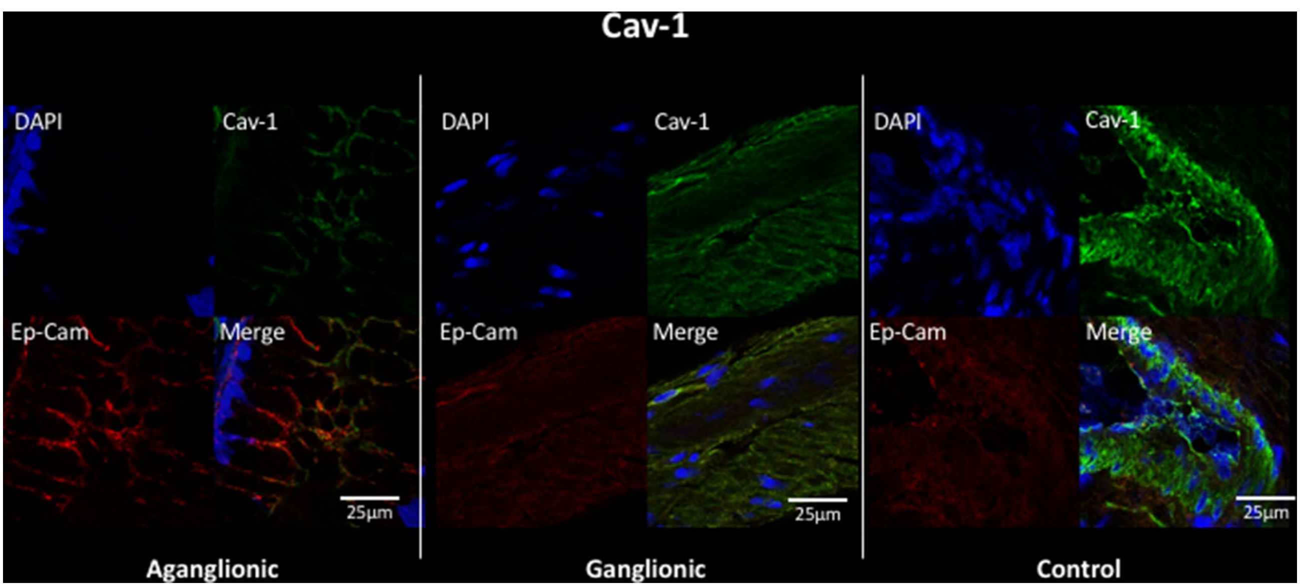

Fig. 2 Cav-1 expression (green) in the colonic epithelium (red) of HSCR specimens compared to normal controls. EpCAM (red) was used to identify colonic epithelium to show coexpression with Cav-1 (scale bar 25 µm, original magnification × 63). Cav-1 expression (green) in the colonic epithelium (red) of HSCR compared to healthy controls. A total of eight specimens were analyzed; only the most representative stainings are shown. Compared with the control group, there was a significantly decreased expression of Cav-1 in colonic epithelium of patients with HSCR

anti-inflammatory effects through the regulation of eNOS [19, 28]. Furthermore, Cav-1 is able to inhibit TNF production through the activation of TLR4 and inhibit TGFβ [19, 29, 30].

Cav-1 is also involved in regulating the function of the different NOS isoforms. Cav-1 inhibits NOS isoforms which catalyze the conversion of arginine and oxygen into NO and citrulline [4, 31]. iNOS expression is increased in an inflammatory state and is responsible for high levels of NO, which dramatically increase blood flow by dilating the capillaries [5]. Sorrells et al. [32] reported that sustained upregulation of iNOS in the intestine caused by LPS can lead to intestinal barrier failure. High levels of NO react with the superoxide ion to form peroxynitrite which may damage the epithelium in multiple ways [16]. It may induce enterocyte apoptosis and inhibit epithelial restitution processes including enterocyte proliferation and migration [33–35].

Our findings show that the ganglionic bowel in the resected pull-through specimens in HSCR, although normal from a neuroanatomical standpoint, is deficient in Cav-1 expression. We speculate that decreased Cav-1 expression may result in overactivity of iNOS, leading to high levels of NO contributing to the epithelial damage seen in HAEC. Four of the ten patients in our study had preoperative enterocolitis. However, Cav-1 expression in the aganglionic and ganglionic bowel was not significantly different from HSCR patients with enterocolitis compared to those who did not have enterocolitis. One limitation of our study is the small number of patients. Future studies comprising of a large number of HSCR patients with preoperative history of HAEC should be investigated for both Cav-1 and iNOS expression in the ganglionic and aganglionic bowel of the pull-through specimens, and this should provide further insights into the pathogenesis of HAEC in HSCR patients.

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