High intestinal vascular permeability in a murine model for Hirschsprung’s disease: implications for postoperative Hirschsprung-associated enterocolitis

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Abstract
Purpose Intestinal vascular permeability (VP) in a murine model for Hirschsprung’s disease (HD) and postoperative Hirschsprung-associated enterocolitis (HAEC) were investigated.
Methods Intestinal VP was determined using a Miles assay using 1% Evans blue injected into a superficial temporal vein of newborn endothelin receptor-B KO HD model (KO) and syngeneic wild-type (WT) mice (n = 5, respectively). Extravasated Evans blue in normoganglionic ileum (Ng-I), normoganglionic proximal colon (Ng-PC) and aganglionic distal colon (Ag-DC) was quantified by absorbance at 620 nm. Quantitative polymerase chain reaction (qPCR) for Vascular Endothelial Growth Factor A (VEGF-A), VEGF-B, CDH5, SELE and CD31, and immunofluorescence for CD31 were performed.
Results VP was significantly higher in Ng-I, Ng-PC, and Ag-DC from KO than WT (p < 0.01, p < 0.05, and p < 0.05, respectively). qPCR demonstrated upregulated VEGF-A in Ng-I and Ag-DC, VEGF-B in Ng-I, and SELE in Ng-I and Ng-PC (p < 0.05, p < 0.05, p < 0.05, p < 0.01 and p < 0.05, respectively), and downregulated CDH5 in Ng-I and Ng-PC from KO (p < 0.05, respectively). Expression of CD31 mRNA in Ng-I and Ag-DC from KO was significantly higher on qPCR (p < 0.05) but differences on immunofluorescence were not significant.
Conclusions VP may be etiologic for postoperative HAEC throughout the intestinal tract even after excision of aganglionic bowel.

Keywords Hirschsprung’s disease · Hirschsprung associated enterocolitis · Endothelin receptor-B · Vascular permeability · Vascular integrity · Vascular endothelial growth factor

Introduction
Hirschsprung’s disease (HD) is characterized by intestinal dysmotility due to an absence of ganglionic cells differentiated from enteric neural crest cells (ENCC). Resection of aganglionic bowel and pulling-down normoganglionic bowel to the anus are performed to normalize bowel function [1]. Hirschsprung associated enterocolitis (HAEC) causes morbidity and can be serious enough to cause death in HD patients both before and after definitive pull-through surgery [2]. While its etiopathogenesis remains unknown, hypoperistalsis due to a lack of ganglionic cells has been conventionally regarded as responsible for the development of HAEC. However, its incidence can range from 15–50 to 2–33% for pre and postoperative HAEC, respectively [3], suggesting a more generalized cause involving the entire intestinal tract rather than a nerve cell distribution anomaly causing localized aganglionic bowel [4].

In addition to ganglion and nerve cell anomalies that are pathognomonic for HD, altered vascular density has also been reported in the gastrointestinal tract (GIT) of HD patients [5]. For example, increased submucous microvascular density has been observed in aganglionic colon from HD patients compared with ganglionic colon [5] and vascular...
networks comprised of a variety of fluid and blood-conducting vessels with the overall goal of perfusing all metabolic tissues of the intestines are disrupted with significantly decreased blood vessel density in the distal colon of rearranged during transfection (RET) knockout mice, an animal model for HD compared with controls [5]. These findings may just be the result of aberrant neovascularization and their specific clinical importance is unclear but their association with HD is relevant.

Vascular endothelial growth factor A (VEGF-A) mediates angiogenesis and is also involved in the etiopathology of several diseases such as inflammatory bowel, chronic inflammatory, and autoimmune diseases [6]. Tissue vascular permeability (VP) can be enhanced by VEGF-A activation and this mechanism is closely linked to serious inflammatory conditions such as inflammatory bowel disease due to disrupted endothelial barrier function [6–9]. In addition, abnormal vascularity caused by irregular VEGFA-induced angiogenesis has increased permeability and susceptibility to invasive cancer cells [10, 11]. Despite these known actions, there are no reports of increased VP in the GIT of HD patients.

The status of abnormally permeable vessels and aberrant angiogenesis-mediated stimuli observed in the GIT of Endothelin receptor-B null mice (KO), a representative murine model for HD presenting with distal colorectal aganglionicosis [12] was investigated as a possible cause for HAEC. Although HAEC can also arise preoperatively, bowel dysmotility associated with HD affects its clinical presentation. Postoperative HAEC arises in the absence of aganglionic bowel, allowing the etiology of HAEC to be investigated more specifically. As a result, this study focuses on postoperative HAEC because it is more distinct clinically than preoperative HAEC, although the morbidity involved is essentially the same.

**Material and methods**

**Animal model**

KO mice were originally obtained from Jackson Laboratory (Bar Harbor, USA). Homozygous KO mice were raised at Juntendo University School of Medicine by crossing pairs of heterozygous littermates. Genotypes were determined by polymerase chain reaction (PCR). Newborn homozygous KO mice and syngeneic wild-type (WT) mice obtained within 24 h of birth were used to minimize the effect of growth related inflammation that might occur. Approval for this study was obtained from the Animal Care and Use Committee (registration number: 1570, permission number: 2022280) at Juntendo University School of Medicine. GIT specimens studied were normoganglionic ileum (Ng-I), normoganglionic proximal colon (Ng-PC), and aganglionic distal colon (Ag-DC) from KO mice, and ileum (I), proximal colon (PC), and distal colon (DC) from WT mice.

**The miles assay**

VP was determined using the Miles assay [13]. Briefly, 30 μL of 1% Evans blue dye solution dissolved in phosphate-buffered saline (PBS) was injected into a superficial temporal vein just anterior to the ear bud of neonatal KO and WT mice using a 31-gauge needle under stereomicroscopic control (n = 5, respectively). Mice were perfused with 200 μL of PBS through the left ventricle 2 h after injection. GIT specimens (Ng-I, Ng-PC, Ag-DC from KO and I, PC and DC from WT as well) were harvested from mice sacrificed with carbon dioxide, cleaned, and immediately placed in a centrifuge tube containing 1.5 mL of formamide. After incubation for 24 h at 60 °C, the formamide extract was collected, and colorimetric measurements performed with a Nano Drop 1000 spectrophotometer (ND-1000; Thermo Scientific, Yokohama, Japan) at 620 nm. The Evans blue content of extracts was quantified with a standard curve.

**RNA isolation from gut, complementary DNA (cDNA) synthesis and quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted from GIT specimens taken from subject mice [14]. Real-time PCR (RT-PCR) was performed for VEGF-A, VEGF-B, endothelial integrity-associated genes such as SELE and CDH5, and CD31, a marker for endothelial cells, using the 7500 Fast RT-PCR system (Applied Biosystems, Forster City, Calif), according to the manufacturer’s specifications. All results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to compensate for differences in the amount of cDNA.

Sequences of primers were as follows: Forward 5′ GAC ATCAAGAGGTGGTGAGCAG 3′ and Reverse 5′ ATA CCAGGAATGACCTTGACAAA 3′ for GAPDH; Forward 5′ CACTGCTTTGGGAGCCTTC 3′ and Reverse 5′—GGG GCAGCGATTCATTATTTCT—3′ for CDH5; Forward 5′ ATG CCTCGGGCTTCTCTC 3′ and Reverse 5′—GTAGTCCGG CTCGACATTGC—3′ for SELE; Forward 5′ AGGGAGACT ATCCAGCGGAC 3′ and Reverse 5′—CCAACCTCCTCA AACCGTGT—3′ for VEGFA; Forward 5′ GCCAGACAG GGTGCCCATAC 3′ and Reverse 5′—GGAGTGGGATGG ATGATTCAG—3′ for VEGFB; Forward 5′ AACAGAAAC CCGTGGAGATG 3′ and Reverse 5′—GTCTCTGTGGCT CTCGTTC—3′ for CD31.
**Immunofluorescence, confocal microscopy, and image quantification**

GIT specimens were fixed in 4% paraformaldehyde, embedded in OCT Mounting Compound (VWR International, Leuven, Belgium), frozen at −80 °C, sectioned transversely at a thickness of 5 μm and mounted on Superfrost Plus slides (VWR International). Preparations were washed three times in PBS + 0.5% Triton X-100 (PBST). Primary antibody to CD31 (Rabbit, Abcam, 1:1200) was diluted in blocking solution (PBST + 5% BSA) and slides were incubated with primary antibody at 4 °C overnight. After several washes with PBS, a secondary antibody was added in the blocking solution for 1 h at room temperature at the following dilution: anti-rabbit Alexa Flour 488 (Invitrogen, UK) 1:200. Finally, slides were washed with distilled water and mounted with coverslips using Vectashield containing DAPI (Vector Laboratories, USA). Images were acquired on a confocal laser-scanning microscope (LSM780, Zeiss, Germany). Immunostaining was repeated on at least 3 tissue sections per tissue block. Only representative immunostainings are presented in this report.

CD31 fluorescence was quantified by calculating corrected total cell fluorescence (CTCF) using the formula: CTCF = integrated density–area of selected cell x mean fluorescence of background readings) using image J software.

**Statistical analysis**

Differences between two groups were tested using the unpaired t-test. All statistical tests were two-sided and all data were expressed as mean ± standard deviations.

A p-value of 0.05 or less was considered statistically significant.

**Results**

**VP was higher in the GIT of neonatal KO mice**

Extravasated Evans blue was observed macroscopically in harvested GIT specimens from KO mice using the Miles assay (Fig. 1a). Quantifying the amount of extravasated Evans blue by absorbance at 620 nm demonstrated significant differences for Ng-PC and Ag-DC and particularly Ng-I, indicating increased VP in KO mice (Fig. 1b). Interestingly, the results for extravasated Evans blue were similar for PC and DC from WT mice, and also Ng-PC and Ag-DC from KO mice, suggesting that these findings are independent of whether bowel was normoganglionic or aganglionic.

**Expression of permeability associated gene was altered in KO mice**

Relative mRNA expression was assessed by qPCR for CDH5, a representative marker for vessel integrity that can be downregulated in permeable endothelial cells [15], and SELE, an endothelial adhesion molecule, that can be activated in highly permeable endothelial cells [16]. A significant decrease in CDH5 mRNA (Fig. 2a) and increase in SELE mRNA (Fig. 2b) was observed in Ng-I and Ng-PC from KO mice compared with WT mice, which was compatible with increased VP especially in Ng-I from KO mice. In contrast, mRNA expression of CDH5 and SELE were similar between Ng-PC and Ag-DC from KO mice, and in

![Fig. 1](image-url)  
A Typical views of harvested intestines. Extravasated Evans blue is more readily observed macroscopically KO mice compared with WT mice. Arrow heads indicate the cecum.

![Fig. 1](image-url)  
B Summary of intestinal vascular permeability by the Miles assay in neonatal mice. Quantitatively increased absorbance of extravasated Evans blue at 620 nm in KO mice for Ng-I, Ng-PC, and Ag-DC (**p < 0.01, and *p < 0.05)**
PC and DC from WT mice (Fig. 2c, d), indicating that these altered gene expressions also appear to be independent of whether bowel was normoganglionic or aganglionic.

Expression of angiogenesis promoting gene was activated in the intestine of KO mice

qPCR for VEGF-A/B to test whether increased VP was associated with aberrant expression of major angiogenesis-mediating factors in the GIT of KO mice. qPCR showed significantly increased mRNA expression of VEGF-A in Ng-I and Ag-DC (Fig. 3a) and VEGF-B in Ng-I (Fig. 3b) in KO mice. Expression of both substances in KO mice was relatively higher than in WT mice, but differences were not statistically significant compared with other GIT specimens. The mRNA expression of VEGF-A and VEGF-B were similar for Ng-PC and Ag-DC from KO mice, and in PC and DC from WT mice (Fig. 2c, d), indicating that these data were independent of whether bowel was normoganglionic or aganglionic.

Distribution of endothelial cells in the GIT did not differ between KO and WT mice

The extent of angiogenesis induced by activated VEGF-A/B signals was assessed using expression of CD31 in KO mice detected by qPCR and tissue immunofluorescence. As expected, CD31 mRNA was higher in Ng-I and Ag-DC of KO mice than WT mice (Fig. 4a). However, the distribution of CD31 + cells was similar or slightly increased in entire regions of KO mice on tissue immunofluorescence (Fig. 4b). Despite the relative increase observed in KO mice, differences were not statistically significant when quantified by CTCF calculation (Fig. 4c).
Discussion

Our data demonstrated increased intestinal VP associated with activated VEGF-A/B in KO mice. The mechanism for VEGF-mediated VP has been broadly accepted as an exacerbating factor in a variety of diseases such as Covid-19 induced pneumonia and inflammatory bowel disease [6, 17–19]. These findings may indicate a potential etiopathologic role in the development of HAEC. Of note is that the amount of extravasated Evans blue and expression of SELE, CDH5, and VEGF-A/B in were not affected by whether bowel had normal or abnormal ganglion distribution in KO mice; in other words irrespective of whether bowel was ganglionic or aganglionic, extravasation was different based on the type of mouse. This indicates that the mechanism by which HAEC may be induced in association with increased VP will be activated even after resection of aganglionic bowel which suggests that increased VP with activated VEGF-A/B is caused by the genetic loss of endothelin receptor-B in KO mice rather than direct association with neural abnormality due to dysfunctional ENCC in this mouse model.

The primary role of endothelin receptor-B signaling is to inhibit the differentiation of ENCC and to maintain them in a proliferative state [20]. Endothelin receptor-B is also expressed in the endothelial cells of several tissues and plays an important role in the control of vascular tone by causing constriction [21]. Carpenter et al., reported that loss of endothelin receptor-B predisposed pulmonary endothelial cells to high permeability in rats, via increased VEGF expression induced by secondarily elevated endothelin-1 peptide [22], a similar situation to the findings of this study. In another study using cultured rat cardiac microvascular endothelial cells in vitro, endothelial junction integrity determined by transendothelial electrical resistance was not changed by an endothelin receptor-B blocker [23]. The
effect of endothelin receptor-B on endothelial permeability through inactivation might be diverse and depend on various factors such as signal alteration, environment (in vitro or in vivo), or type of tissue.

Higher CD31 mRNA and relatively increased distribution of CD31 protein signal was observed in the GIT of KO mice but differences were not statistically significant. Although there was induction by upregulated VEGF-A/B, it was ineffective for excessive angiogenesis. Nevertheless, upregulated VEGF-A/B in KO mice was sufficient to promote GIT VP [24]. Carpenter et al. also reported that overexpressed endothelin-1 in Endothelin receptor-B KO could still act via endothelin receptor-A to stimulate VEGF mRNA and protein which supported the findings of the present study [22]. In contrast, Schrenk et al. reported their observation of degraded density of intestinal vessels with disorganized structure in RET KO HD model mice [5], interesting because the resultant aberrant vascular formation was opposite in a different murine model for HD. Thus, there may be other mechanisms for causing HAEC involving the induction of distinct genetic features.

Increased SELE mRNA and decreased CDH5 mRNA shown by qPCR in association with enhanced VP seen in KO mice was consistent with published reports [7, 15]. Especially in oncology, expression of endothelin receptor-B in human hepatocellular and lung carcinoma was positively correlated with CDH5 expression [25, 26] and in the lungs of smokers who underwent lung resection for lung cancer or transplantation for advanced chronic obstructive pulmonary disease [27], endothelin receptor-B was significantly downregulated whereas there was a twofold increase in the expression of SELE. Collectively, endothelin receptor-B appears to be an adequate mediator maintaining vascular integrity by regulating these substances.

**Conclusions**

In the present study, experimental murine model evidence was instrumental for demonstrating increased GIT VP with altered expression of VEGF irrespective of whether the bowel was normoganglionic or aganglionic or whether the bowel had been resected or not. This observation is reported for the first time and may contribute to causing HAEC. Of note, some 5% of HD patients have a documented alteration of the endothelin receptor-B gene [28]. Further research...
on the biological effect of this mechanism and its implication for causing HAEC and its severity is planned and an enterocolitis model evaluating endothelial barrier function targeting etiopathogenesis for potential clinical application is being developed.

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Author contributions KS, SY, and KM designed the study. NF, SS, SN, and AY provided the conceptual advice. KS, SY, KM, SK, KA, and SN performed experiments. KS, SY, KM, and SN analyzed the data. KS, KM, GL, and AY wrote the manuscript.

Data availability statement Data are available on reasonable request from the authors.

Declarations

Conflict of interest The authors declare no conflict of interest.

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