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CXCL5 is Required For Angiogenesis, but not Structural Adaptation after Small Bowel Resection

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Abstract

Purpose: Intestinal adaptation is the compensatory response to massive small bowel resection (SBR) and characterized by lengthening of villi and deepening of crypts, resulting in increased mucosal surface area. Previous studies have demonstrated increased villus capillary blood vessel density after SBR, suggesting a role for angiogenesis in the development of resection-induced adaptation. Since we have previously shown enhanced expression of the pro-angiogenic chemokine CXCL5 after SBR, the purpose of this study was to determine the effect of disrupted CXCL5 expression on intestinal adaptation.

Methods: CXCL5 knock-out (KO) and C57BL/6 wild type (WT) mice were subjected to either a 50% proximal SBR or sham operation. Ileal tissue was harvested on postoperative day 7. To assess for adaptation, villus height and crypt depth were measured. Submucosal capillary density was measured by CD31 immunohistochemistry.

Results: Both CXCL5-KO and WT mice demonstrated normal structural features of adaptation. Submucosal capillary density increased in the WT but not in the KO mice following SBR.

Conclusion: CXCL5 is required for increased intestinal angiogenesis during resection-induced adaptation. Since adaptive villus growth occurs despite impaired CXCL5 expression and enhanced angiogenesis, this suggests that the growth of new blood vessels is not needed for resection-induced mucosal surface area expansion following massive SBR.

Keywords

CXCL5; Adaptation; Small bowel resection; Intestine; Angiogenesis

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INTRODUCTION

Short gut syndrome results from substantial intestinal loss and is a condition of high morbidity and mortality within the pediatric population. Following massive small bowel resection (SBR), intestinal adaptation is a critical, compensatory response in both humans and animal models that allows for adequate absorption of enteral nutrition despite significant loss of bowel length [1-3]. This phenomenon is characterized by significant increases in villus height and crypt depth, in part due to increased enterocyte proliferation, and resulting in increased absorptive mucosal surface area to compensate for the attenuated bowel length [1].

Angiogenesis is the growth of new blood vessels and is known to play a significant role in general states of cellular proliferation [4, 5]. Within the intestine, supplementation of proangiogenic growth factors has been shown to enhance intestinal mucosal growth [6]. Alternatively, inhibition of vascular endothelial growth factor (VEGF) resulted in a decreased adaptive response following intestinal loss [7]. We have previously reported increased villus capillary density during the intestinal adaptation response to massive SBR [8]. This increased villus capillary density in the intestine is preceded by an increase in the intestinal gene expression of proangiogenic chemokine ligand 5 (CXCL5) [8, 9].

CXCL5 belongs to the CXC chemokine family of molecules that bind the CXCR2 receptor and serve as neutrophil chemoattractants and promoters of neovascularization [10-12]. CXCL5 has been demonstrated to play a role in neutrophil homeostasis at mucosal sites, including the intestine [13]. In patients with inflammatory bowel disease, CXCL5 has been shown to be overexpressed in the intestinal epithelial cells [14, 15]. CXCL5 expression is enhanced in murine models of colitis and inhibition of CXCL5 attenuates disease severity [16, 17]. As well, CXCL5 expression has been shown to be upregulated in human necrotizing enterocolitis (NEC) intestinal tissue [18]. In murine neonatal models of NEC, CXCL5 has been shown to recruit macrophages to the gastrointestinal tract during inflammatory mucosal injury [18].

Since we have previously shown increased intestinal expression of CXCL5 after SBR [8, 9], the purpose of the present study was to determine the effects of absent CXCL5 expression on structural features of intestinal adaptation as well as angiogenic responses to massive SBR.

MATERIALS AND METHODS

Experimental design

A protocol for this study was approved by the Washington University Animal Studies Committee (Protocol #20100103) and in accordance with the National Institute of Health laboratory animal care and use guidelines. Four experimental groups were studied: wild type (WT) mice that underwent sham operation (n=9) or 50% proximal SBR (n=6), and CXCL5 knock-out (KO) mice that underwent sham operation (n=10) or 50% proximal SBR (n=13). CXCL5 gene deletion was confirmed via RT-PCR of CXCL5 mRNA within the small intestine. Ileal tissue was harvested in all mouse groups on post-operative day 7. To assess

for adaptation, villus height and crypt depth were measured via hematoxylin and eosin (H&E)-stained histology. In addition, submucosal capillary density was measured by CD31-immunohistochemistry.

Animals

CXCL5 KO mice on a C57BL/6 background were generously provided by Junjie Mei and Scott Worthen, Children's Hospital of Philadelphia (Philadelphia, Pennsylvania) [19]. Non-mutant C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were used as WT controls. Male and female mice aged 7-15 weeks were used in this study with a weight range of 23.5 to 29.5 g (WT) and 18.0 to 28.0 g (KO). Mice were kept on a 12-hour light-dark schedule and were housed in a standard facility. The mice were given a liquid rodent diet (Micro-Stabilized Rodent Liquid Diet LD101; Purina Mills, St Louis, MO) 1 day prior to surgery.

Operative technique

Mice underwent 50% proximal SBR or sham operation (transection and reanastomosis only) as previously described [1]. Briefly, mice that underwent SBR had transection of the bowel at a point 12 cm proximal to the ileal-cecal junction and also at a point 1 to 2 cm distal to the ligament of Treitz. The mesentery was ligated and the intervening bowel was removed. Intestinal continuity was restored with an end-to-end anastomosis using 9-0 monofilament suture. In mice undergoing sham operation the bowel was transected at a point 12 cm proximal to the ileal-cecal junction and intestinal continuity was restored with an end-to-end reanastomosis. Following the operation, mice were provided free access to water for the first 24 hours and then given a liquid rodent diet until sacrifice.

Tissue harvest

On the seventh postoperative day, the mice were anesthetized with a subcutaneous injection of ketamine, xylazine, and acepromazine (4:1:1). A midline laparotomy was performed and the small bowel was flushed with ice-cold phosphate-buffered saline and excised. The first 1 cm segment of bowel distal to the anastomosis was discarded. The next 2 cm segment of bowel was fixed in 10% neutral-buffered formalin for histology. Following tissue harvest, the animal was sacrificed via cervical dislocation.

RT-PCR confirmation of disrupted CXCL5 mRNA expression in enterocytes

Total RNA was extracted from ileal tissue following the manufacturer's protocol for the RNeasy kit (Qiagen, Austin Texas) and total RNA concentration determined spectrophotometrically. Quality of obtained RNA was evaluated using the Bio-Rad Experion System with an RNA StdSens Chip and reagents (Bio-Rad Laboratories, Richmond, CA). A TaqMan RNA-to Ct 1-Step kit (Applied Biosystems, Foster city, CA) was used per the manufacturer's protocol to determine relative gene expression directly from the isolated RNA. Equal amounts of RNA were used for real-time PCR with B-actin as endogenous control and a standard whole bowel sample used as calibrator. CXCL5 gene expression was examined using primers for CXCL5, reagents and with a 750 Fast Real-Time PCR instrument all from Applied Biosystems (Foster City, CA).

Structural adaptation measurements

Villus height and crypt depth were measured on H&E stained sections by two independent investigators blinded to mouse strain and procedure using the image analysis software MetaMorph (Dowington, PA). At least twenty well-oriented villi and crypts per animal were measured.

Submucosal capillary density measurements

Slides were deparaffinized in xylene and rehydrated in gradients of ethanol. Antigen retrieval was carried out using a pressure cooker that reaches 99 C at 18 PSI for 3 minutes in DIVA solution (BioCare, Bedford Park, IL).

Primary antibody to CD31 (Abcam, Cambridge, MA) was diluted to 1:400 in DaVinci Green (BioCare, Bedford Park, IL) and incubated overnight at 4°C. Slides were incubated at room temperature (RT) for 30 minutes with a conjugated polymer probe diluted 1:4 in PBS with Tween 20 (PBST). HRP conjugated probe (Mach 2 Rabbit HRP, BioCare, Bedford Park, IL) was diluted in PBST (BioCare, Bedford Park, IL) to 1:4 and incubated for 30 minutes at RT. The slides were developed with 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) for permanent visualization, washed in distilled water, and counterstained with hematoxylin.

CD31-stained microvessels in the submucosal area of the small intestine were counted under light microscopy at $\times 40$ magnification by two independent investigators blinded to mouse strain or procedure. At least 10 well-oriented fields per animal were counted and the results from the two investigators averaged for each animal.

Statistical analysis

Values are presented as mean $\pm$ SEM. The Sigma Stat statistical package (SPSS, Chicago, IL) was used for all statistical analyses. Student's t-test was used for comparisons of two groups while ANOVA was used for comparisons of more than two groups. A p-value of less than 0.05 was considered significant.

RESULTS

Intestinal Adaptation in CXCL5 KO Mice

RT-PCR of mRNA isolated from the small intestine from CXCL5 KO and WT mice confirmed that CXCL5 mRNA was absent only in the KO mice (data not shown). Structural adaptation occurred normally in both the WT and CXCL5 KO mice that underwent SBR, as evident by deeper crypts (WT— 92.7 ± 5.2 vs. 114.8 ± 4.1 ; KO— 87.9 ± 3.9 vs. 105.9 ± 5.4 ; $p < 0.05$) and taller villi (WT— 217.4 ± 6.8 vs. 297.1 ± 7.4 ; KO 204.0 ± 10.6 vs. 315.3 ± 11.3 ; $p < 0.05$) between sham and SBR groups [Figures 1 and 2]. Equivalent degrees of morphological adaptation were noted between WT and KO groups after SBR.

Submucosal capillary density in CXCL5 KO Mice

The submucosal capillary density for WT mice increased significantly following SBR (6.7 ± 0.3 vs. 8.3 ± 0.4 , $p < 0.05$). However, there was no change in submucosal capillary

density for CXCL5 KO mice following SBR (6.8 ± 0.2 vs. 6.5 ± 0.2 , $p < 0.05$) [Figures 3 and 4]. Further, CXCL5 KO mice demonstrated a significantly lower submucosal capillary density as compared to WT mice after SBR.

DISCUSSION

In the present study, we verified that SBR results in adaptive increases in villus height and crypt depth as well as an angiogenic response in the intestinal submucosa. We also confirmed that CXCL5 expression was required for angiogenesis, but not for the morphological alterations associated with intestinal adaptation. These findings would suggest that angiogenesis is not a necessary element for structural adaptive changes in the remnant intestinal mucosa.

Angiogenesis represents the formation of new capillaries from preexisting capillary networks and involves the activation of endothelial cells. Endothelial cell proliferation is critical for several physiologic conditions, including endometrial proliferation, recovery from ischemic injury, and wound healing [20-22]. Recently, we discovered evidence for enhanced blood vessel growth within the central core of adapting villi after massive SBR [8]. The significance of angiogenesis for normal adaptation responses is presently unknown although in a prior study, we found that inhibition of angiogenesis by selective blockade of salivary-derived vascular endothelial growth factor (VEGF) resulted in impaired adaptation responses [7].

It is possible that the stimulus for enterocyte proliferation and increased villus growth during resection-induced adaptation requires increased blood flow to provide greater nutrient and oxygen delivery. As such, angiogenesis would be a secondary response to the stimulus for mucosal growth. This concept would be supported by our prior studies in which villus growth occurred prior to the histologic evidence for new blood vessel growth [8]. On the other hand, we have recently identified immediate (within minutes) reductions in intestinal blood flow and enhanced expression of hypoxia-inducing factor- α (HIF1 α) after intestinal resection [23]. HIF1 α is a transcription factor noted to stimulate the expression of multiple proangiogenic growth factors [24]. These data would suggest that the angiogenic response is a primary stimulus for enhanced enterocyte proliferation and villus growth. The findings of this study would support the notion that the responses of angiogenesis and mucosal growth to massive intestinal resection are uncoupled. This finding contrasts with the observation that angiogenesis is required for liver regeneration after partial hepatectomy [25].

We have shown that angiogenesis in response to SBR appears to require CXCL5 expression. The expression of this chemokine has been shown to be elevated within the gut mucosa under conditions of angiogenesis associated with intestinal inflammation [26]. We have not identified gross evidence for inflammation in the intestine of our animals undergoing SBR. We have, however previously localized increased expression of CXCL5 to the endothelium within the villus core after SBR [9]. The mechanism for enhanced CXCL5 expression is presently unknown, but may involve signaling via the epidermal growth factor receptor (EGFR) since transgenic EGF overexpressing mice were found to have significantly higher villus core CXCL5 levels compared with non-transgenic mice [9]. Further, in this same

study, we found that treatment of human umbilical vein endothelial cells with EGF also induced the expression of CXCL5. Although normal morphologic features of adaptation may not require CXCL5 expression and angiogenesis as revealed in the current study, CXCL5 may be required for the enhanced adaptation responses associated with EGFR stimulation [27]. Along these lines, heparin-binding EGF-like growth factor (HB-EGF) is another EGFR ligand and has been shown to enhance intestinal blood flow in a murine model of necrotizing enterocolitis [28]. Alternatively, disruption of HB-EGF expression is associated with impaired intestinal angiogenesis [29].

In this study, we have shown that blood vessel growth in the intestinal submucosa in association with SBR was blunted in the CXCL5-KO mice. This site of angiogenesis is different than our previous work whereby we demonstrated new blood vessel growth within the villus core after SBR [8]. In that study, we injected high-molecular weight fluorescein isothiocyanate/dextran into the retro-orbital plexus of mice prior to harvesting the intestine. Capillary density was then measured using confocal microscopy. We did not follow the same protocol for this study since our initial intent was to determine the effect of absent CXCL5 expression on structural adaptation. After we determined that adaptation occurred normally, we subsequently employed CD31 immunostaining on the same tissue blocks in order to quantify capillary density in the submucosa. Although this is a different site for measurement, the fact that we identified increased capillary density after SBR in the WT mice would suggest that this location is a reasonable surrogate for the angiogenesis that is taking place in the villus core.

Although we have demonstrated that increased angiogenesis is not essential for mucosal growth after SBR, we have yet to investigate the functional consequences of this perturbed response. Postprandial gut mucosal hyperemia is a recognized, normal response to feeding and defined as an increase in blood flow to the gastrointestinal tract to aid in the absorption and digestion of luminal nutrients [30]. It is therefore possible that the growth of new capillaries and enhanced blood flow after massive intestinal loss is a critical response to maximize digestion and absorption. Although we did not observe differences in weight gain between the CXCL5-KO and WT mice after SBR, studies of specific absorption and digestion were not done and might have revealed important perturbations.

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Figure 1.

Adaptation occurs normally after 50% proximal small bowel resection (SBR) in both wild-type (WT) and CXCL5 knockout (KO) mice. Hematoxylin and eosin (H&E)-stained section of mouse ileum (magnification $\times 10$) – A: WT sham operation. B: WT 50% proximal small bowel resection (SBR). C: CXCL5-KO sham operation. D: KO 50% proximal SBR with complete villus and crypt adaptive response.

Figure 2.

Crypt depth and Villus height changes in response to 50% proximal small bowel resection (SBR) – Wild type (WT) and CXCL5 knock-out (KO) mice underwent either 50% SBR or sham operation (transection and reanastomosis of the bowel alone). On post operative day 7, the ileum was harvested and crypt depth and villus height were measured from H&E-stained sections. * = $p < 0.05$.

Figure 3.

CD31-immunostaining of mouse ileum (magnification $\times 40$) – A: Wild type (WT) sham operation. B: WT 50% proximal small bowel resection (SBR). C: CXCL5 knock-out (KO) sham operation. D: KO 50% proximal SBR. Arrows represent CD31+ stained blood vessels within the submucosa.

Figure 4.

Quantification of submucosal capillary density – Wild type (WT) and CXCL5 knock-out (KO) mice underwent either 50% proximal small bowel resection (SBR) or sham operation (transection and reanastomosis of the bowel alone). On post operative day 7, the ileum was harvested and submucosal capillary density was measured from CD31-immunostained sections. * = $p < 0.05$.