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Epithelial IGF1R is dispensable for IGF2 mediated enhanced intestinal adaptation in retinoblastoma-deficient mice☆

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ABSTRACT

Purpose: Previously, we demonstrated enhanced adaptation after small bowel resection (SBR) in intestinal-specific retinoblastoma (Rb)-deficient mice along with elevated levels of insulin-like growth factor 2 (IGF2) expression within the villi. The purpose of this study was to verify that the insulin-like growth factor 1 receptor (IGF1R) plays a role in this phenomenon.
Methods: Inducible and intestinal specific Rb and IGF1R double knockout mice (iRb/IGF1R-IKO) (n = 4) and Rb single knockout mice (iRb-IKO) (n = 5) underwent 50% mid SBR. On post-operative day 28, mice were harvested, and structural adaptation was measured as changes in crypt depth and villus height. Rates of enterocyte proliferation were recorded. IGF2 expression within the remnant villi was measured via RT-PCR.
Results: Both iRb-IKO and iRb/IGF1R-IKO mice demonstrated enhanced adaptation with at least a 45% increase in both crypt depth and villus height in the proximal and distal remnant bowel. Both groups showed elevation of IGF2 expression in the remnant villi, but there were no differences between the two groups.
Conclusion: Epithelial IGF1R is dispensable for IGF2-mediated enhanced intestinal adaptation in retinoblastoma-deficient mice. Our findings suggest that IGF2 signals for enhanced adaptation in cells outside of the epithelium. Further investigation is needed to study the IGF2/IGF1R signaling interaction within the mesenchyme.
Level of Evidence: Animal study - not clinical.

Short gut syndrome is a morbid clinical condition that follows a massive small bowel resection (SBR) required to treat several pediatric conditions such as necrotizing enterocolitis, midgut volvulus, and complicated gastroschisis [1]. Intestinal adaptation is the response to massive SBR and is characterized by structural increases in crypt depth and villus height resulting in increased absorptive and digestive mucosal surface area [2,3]. The exact mechanisms for adaptation are incompletely understood and continue to be an area of research with the intent to achieve enteral autonomy in short gut patients.

Insulin-like growth factor 2 (IGF2) is a recognized intestinotrophic factor which binds and signals through insulin-like growth factor 1 receptor (IGF1R) [4]. Studies have suggested that infusion of such intestinotrophic factors have an exaggerated adaptation response [5–10]. Previously, our lab demonstrated that disrupting the expression of retinoblastoma protein (Rb) within the enterocytes of mice resulted in taller villi [11]. Furthermore, we discovered that IGF2 expression was significantly elevated within the villi of the Rb-null mice [12,13]. The deletion of enterocyte Rb in the setting of SBR resulted in even taller villi and deeper crypts thereby enhancing adaptation. This phenomenon of enhanced adaptation was prevented when IGF2 expression was deleted. Therefore, IGF2 is considered to be a necessary intestinotrophic factor for Rb-mediated enhanced adaptation after SBR [13].

We have previously demonstrated that disrupting the expression of growth factor receptors such as IGF1R within the intestinal epithelium has no effect on the degree of intestinal adaptation after SBR [14,15]. We also learned that IGF2 by itself is not required for normal adaptation to occur. These findings suggest that IGF1R may play a more important role in the sub-epithelial compartments such as muscularis or perhaps that there may be other compensatory mechanisms which cause other pathways to be upregulated during the process of intestinal adaptation when the expression of an epithelial-targeted growth factor receptor is knocked out.

Given our previous findings, we sought to determine whether IGF1R within the enterocytes is necessary for enhanced adaptation to occur

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after SBR in the setting of Rb deletion knowing that IGF2 is elevated and the driving force behind Rb-mediated adaptive growth. To investigate this, we crossed Rb (flox/flox) and IGF1R (flox/flox) mice with the Villin-Cre ER mouse line to create an Rb/IGF1R double knockout mouse line.

1. Materials and methods

All of our protocols and experiments were approved by the Washington University Animals Studies Committee (Protocol #20130038) and strictly followed National Institutes of Health (NIH) animal care guidelines.

1.1. Animals

Previously, we obtained mice with a tamoxifen-inducible Cre-fusion protein with a villin promoter (Sylvie Robine, Curie institute, Paris, France). We obtained mice with floxed Rb gene and IGF1R gene (Jackson Laboratories, Bar Harbor, ME). We crossed (Villin Cr-ER(+); Rb(f/f)) with (Villin Cr-ER(+); IGF1R(f/f)) mice to create an inducible, intestinal epithelial-specific Rb and IGF1R double knockout mice (iRb/IGF1R-IKO). Double knockout iRb/IGF1R-IKO mice ($n = 4$) were induced by injecting tamoxifen (0.5 mg/day; Sigma, St. Louis, Missouri) intraperitoneally for 3 days after small bowel resection. Intestinal specific single knockout mice (iRb-IKO) ($n = 5$) served as controls. Tamoxifen was prepared as previously described [16]. Mice were kept in the approved animal holding area with a 12 h light–dark schedule and given rodent chow ad lib after weaning.

1.2. Small bowel resection

All mice underwent 50% mid small bowel resection (SBR) in a manner similar to our previously described proximal SBR [2]. A mid-SBR was performed in these mice to determine whether there was an aboral gradient of IGF2 expression along the bowel. The intestinal resections were performed by transecting the bowel approximately 8 cm distal from the ligament of Treitz and at 8 cm proximal from the cecum. The resected segment was approximately an average of 12 cm in length. After this segment of bowel was removed, intestinal continuity was reestablished by an end-to-end primary anastomosis using interrupted 9–0 monofilament sutures. A 2 cm proximal and distal segment of the resected ileum was fixed in 10% neutral-buffered formalin for baseline histology. All mice were fed a liquid rodent diet post operatively. (Microstabilized Rodent Liquid Diet LD101, Purina Mills, St Louis, MO).

1.3. Experimental design

Both proximal and distal portions of the resected bowel were collected at the time of SBR as a baseline control. On post-operative day (POD) 28, the remnant bowel was removed. Histology was collected to compare adaptive structural changes within the crypts and villi against preoperative baseline measurements. Enterocytes were isolated and used for protein analysis to confirm gene deletion and IGF2 mRNA levels.

After 28 days, structural adaptation was assessed by measuring changes in crypt depth and villus height on both the proximal and distal remnant bowel. Intestinal tissue was cut into two sections each, 50 μm apart followed by H&E staining. Paraffin-embedded tissue sections were also immunohistochemically stained with p-histone 3. Rates of enterocyte proliferation were measured and calculated to a proliferation index by counting the number of p-histone 3 positive cells divided by total number of cells per crypt. A minimum of 20 crypts were counted. All histology slides were measured using MetaMorph Computer program (Molecular Devices, Dowington PA). Western blots were used to confirm Rb and IGF1R deletion within the enterocytes. RT-PCR was used to measure the relative level of IGF2 within the villi.

1.4. Harvest and enterocyte isolation

The remnant intestine was harvested on POD 28 as previously reported [17]. Briefly, a midline laparotomy was performed and the entire small intestine was flushed with ice-cold phosphate buffered saline containing protease inhibitors (0.2 nM phenylmethysulfonyl fluoride, 5 μg/mL aprotinin, 1 μM benzamidine, 1 mM sodium orthovanadate, and 2 μM cantharidin; EMD, Gibbstown, NJ). A 2 cm segment of bowel proximal and distal to the anastomosis was fixed in 10% neutral-buffered formalin to compare with intraoperative histology collected at the time of resection. The remainder of the remnant bowel was used to isolate crypt and villus enterocytes as we have previously described [17]. Protein was isolated for further analysis to verify double knockout. RT-PCR was used to analyze the degree of IGF2 expression present within the villus enterocytes.

1.5. Western blotting

Villus and crypt samples were isolated and lysate was prepared as previously described [15]. Protein concentration was determined using RC DC kit (Bio-Rad; Hercules, CA). Proteins were loaded into a gel in equal concentration amounts. Antibodies used in this study included Rb, IGF1R, and lamin B1 (Cell Signaling Technology; Danvers MA). The proteins were detected using Bio-Rad ChemiDoc™ XRS + system with image Lab ™ software (Bio-Rad, Hercules CA).

1.6. Real-time quantitative PCR

RNA was prepared from harvested crypts and villi as previously described [17] and were homogenized in lysis buffer (RNAqueous kit, Ambion, Austin TX). The RNA was extracted according to kit instructions and stored at −80 °C. Total RNA concentration was determined using a NanoDrop Spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington DE). IGF-2 gene expression was determined using primers and reagents from Applied Biosystems and β-Actin was used as the endogenous control. Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City CA) was used to obtain relative RNA expression.

1.7. Statistics

Statistical analysis was performed using Student's t-test to compare the two experimental groups. A p-value <0.05 was considered significant.

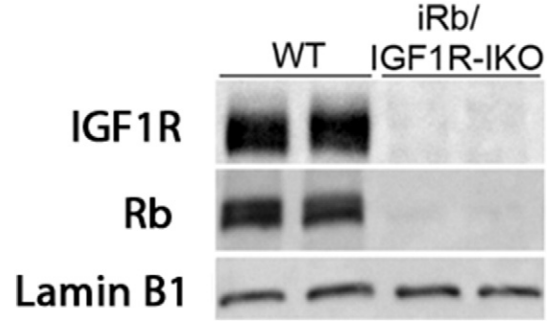

Fig. 1. Western Blot confirming absent retinoblastoma (Rb) and insulin-like growth factor-1 receptor (IGF1R) within enterocytes in induced Rb/IGF1R- intestinal knockout (iRB/IGF1R-IKO) mice. Lamin B1 was used as loading control.