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Insulin-like growth factor 2 and its enterocyte receptor are not required for adaptation in response to massive small bowel resection

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Abstract

Purpose—Enhanced structural features of resection-induced intestinal adaptation have been demonstrated following the administration of multiple different growth factors and peptides. Among these, the insulin-like growth factor (IGF) system has been considered to be significant. In this study, we employ mutant mouse strains to directly test the contribution of IGF2 and its enterocyte receptor (IGF1R) toward the adaptation response to massive small bowel resection (SBR).

Methods—IGF2-knockout (IGF2-KO) (n=8) and intestine specific IGF1R-knockout mice (IGF1R-IKO) (n=9) and their wild type (WT) littermates (n=5, n=7, respectively) underwent 50% proximal SBR. At post-operative day 7, structural adaptation was measured as crypt depth and villus height. Rates of enterocyte proliferation and apoptosis were also recorded.

Results—The successful deletion of IGF2 and IGF1R expression in the enterocytes was confirmed by RT-PCR and Western blot, respectively. Normal adaptation occurred in both IGF2-KO and IGF1R-IKO mice after 50% SBR. Post-operative rates of proliferation and apoptosis in both IGF2-KO and IGF1R-IKO mice were no different than their respective controls.

Conclusion—IGF2 and functional IGF1R signaling in enterocytes are both dispensable for resection-induced adaptation responses. The mechanism for IGF-stimulation of intestinal adaptation may involve other ligands or cellular compartments within the intestine.

Keywords

short gut syndrome; intestinal adaptation; insulin-like growth factor 1 receptor; insulin-like growth factor-2

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INTRODUCTION

Intestinal adaptation is the compensatory response to massive small bowel resection (SBR). This adaptive process is defined structurally by taller villi and deeper crypts which serve to enhance mucosal surface area for improved absorption and digestion [1]. Insulin-like growth factor 1 (IGF1) and insulin-like growth factor 2 (IGF2) are recognized intestinotrophic factors for the small intestine epithelium [2, 3] and signal through insulin-like growth factor 1 receptor (IGF1R). An important role for the IGF system has been suggested by studies in which infusion of IGF1 is associated with an exaggerated adaptation response [4–8]. No prior studies have reported the effects of IGF2 administration in the context of SBR.

Previously, we showed that when Retinoblastoma (Rb) is deleted specifically in the intestine, villus height is markedly increased [9–11]. In the Rb-deficient intestine with expanded villus height, we found that IGF2 expression was dramatically up-regulated in the enterocytes [12]. By breeding Rb-null mice into an IGF2-deficient background, we found that the hyperplastic mucosal phenotype was reversed. Based upon these observations, we propose the hypothesis that IGF2 and IGF1R signaling regulates important adaptive structural responses to massive SBR.

MATERIALS AND METHODS

Experimental design

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

Both IGF2 knock out (IGF2-KO) (n=8) and IGF1R intestine-specific knockout (IGF1R-IKO) (n=9) mouse lines and their wild type (WT) littermates (n=5, n=7 respectively) underwent 50% proximal SBR. Intestinal tissue was obtained at the time of resection for baseline histology. At 7 days after SBR, structural adaptation was measured by measuring changes in crypt depth and villus height. Enterocytes were isolated and used for protein and RNA analysis to confirm gene deletion. Rates of crypt cell proliferation and apoptosis were also analyzed.

Animals and small bowel resection

IGF-2 null mice were generously donated by Dr. Carla Kim, (Department of Genetics; Harvard Medical School). C57BL/6 mice (Jackson Laboratory; Bar Harbor, Maine) were used as wild type controls. For IGF1R-IKO mice, we used an intestine specific tamoxifen inducible (Villin Cre-ER (+); IGF1R (f/f)) recombinant system [13]. Wild type littermates (Villin Cre-ER (-); IGF1R (f/f)) were used as controls. Mice were kept in our standard animal holding area with a 12 hour light-dark schedule and small bowel resection was performed between ages 8 to 10 weeks of age.

Villin Cre-ER (+); IGF1R (f/f) mice and their wild type littermates (Villin Cre-ER (-); IGF1R(f/f)) were injected with tamoxifen for 3 consecutive days. After the series of intraperitoneal injections, the mice were given a 3–5 day rest period prior to SBR. All mice

were fed standard liquid diet (Micro-Stabilized Rodent Liquid Diet LD 101; Purina Mills, St Louis, MO) 24 hours prior to SBR. Mice were given free access to water immediately after surgery and resumed feeding from the standard liquid diet on post-operative day 1. All mice were weighed at the time of resection and also weighed again at the time of harvest on post-operative day 7.

All mice underwent a 50% proximal SBR as previously described [1]. The intestinal resections were done by transecting the bowel approximately 1 to 2 cm distal from the ligament of Treitz and 12 cm proximal from the ileocecal junction with removal of the intervening segment. End-to-end primary anastomosis was done with interrupted 9-0 monofilament sutures. A 2 cm distal segment of the resected bowel was fixed in formalin for baseline histology. Sham operations consisted of transection of the intestine at 12 cm from the ileocecal junction with reanastomosis, but without bowel resection.

Harvest and tissue isolation

All mice were harvested on post-operative day 7 as previously described [9]. The entire small bowel was flushed with ice-cold phosphate buffered saline with protease inhibitors (0.2nM phenylmethanesulfonyl fluoride, 5µg/mL aprotinin, 1µM benzamide, 1mM sodium orthovanadate, and 2µM cantharidin; EMD, Gibbstown, NJ) and excised. A 2 cm segment of bowel distal to the anastomosis was taken starting from 1 cm distal to the anastomosis. This piece of small intestine was fixed in formalin to compare with histology from previously removed bowel. The remainder of the distal segment of bowel was used to isolate crypt and villus enterocyte using our previously published protocol [9]. RNA and protein were isolated for further analysis to verify appropriate genotypes.

Histology

All intestinal sections were cut into two sections each, 50µm apart. Villus height and crypt depth were measured using MetaMorph computer program (Molecular Devices, Downington, PA). At least 20 villi and crypts were measured per animal. Post-operative and intraoperative crypt depth and villus height were compared to calculate a percentage change to determine the magnitude of resection-induced adaptation.

Cell proliferation

At 90 minutes before harvest, 5-bromodeoxyuridine (BrdU; Zymed Laboratories, Inc, San Francisco, CA) was injected intraperitoneally. Rates of proliferation were determined by counting the number of cells staining positive with BrDu divided by the total number of cells in each crypt. A minimum of 20 crypts were counted per histology slide.

Apoptosis index

Each postoperative H&E stained sections were analyzed for apoptotic bodies (pyknotic nuclei, condensed chromatin) [14]. An apoptosis index was measured by counting the number apoptotic bodies found per 50 crypts.

Western blotting

Crypts were isolated and then lysed with sodium dodecyl sulfate (SDS) lysing buffer (50mM Tris-HCL pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 5% mercaptoethanol). The cells were sonicated for 5 seconds and heated in 100°C for 5 minutes. Protein quantification was performed using RC DC kit (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded. Western blot was performed using 8% SDS-PAGE gel probing for IGF1R using anti-IGF1R antibody (Cell Signaling Technology, Danvers, MA) and Lamin B1 using anti-Lamin B1 antibody (Biovision Inc., San Francisco, CA).

RT-PCR

RNA was isolated as previously described [9, 12]. Total RNA was extracted from the intestinal tissue by following the manufacturer's protocol for the RNAqueous kit (Ambion, Austin Texas). Total RNA concentration was determined spectrophotometrically. The quality of the 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 an endogenous control and a standard whole bowel sample used as the calibrator. IGF2 gene expression was examined using primers, reagents and with a 750 Fast Real-Time PCR instrument all from Applied Biosystems (Foster City, CA).

Statistical analysis

Student's t-test was done to compare percent postoperative changes in villus height and crypt depth and also to compare rates of proliferation and apoptosis between groups. A p-value of less than 0.05 was considered to be significant.

RESULTS

Confirmation of enterocyte IGF2 and IGF1R knockout

The successful deletion of IGF1R protein in the small intestine was confirmed by Western blot (Figure 1). Absent IGF2 mRNA expression was confirmed by RT-PCR [12]. The intestine-specific IGF1R knockout mice (IGF1R-IKO) have been characterized previously [15] and neither baseline crypt depth nor villus height were affected. We also verified that there was no phenotypic difference in crypt depth or villus height in unoperated IGF2-KO.

Structural adaptation in IGF2 and IGF1R enterocyte knockout mice

IGF2 null mice had an average $30.0 \pm 7.1\%$ increase in crypt depth and an average of $33.7 \pm 5.9\%$ increase in villus height after SBR when compared with C57BL/6 average of $23.0 \pm 5.2\%$ increase in crypt depth and $48.0 \pm 8.5\%$ in villus height (Figure 2A). There was no statistical difference in the degree of adaptation between the C57BL/6 mice and IGF2 nulls. Wild type littermates had equivalent adaptation with an average of $20.7 \pm 4.3\%$ versus $23.4 \pm 10\%$ increase in crypt depth and an average of $31.5 \pm 4.1\%$ versus $34.5 \pm 5.1\%$ in villus height respectively (Figure 2B). There was also no statistical significance in the degree of

adaptation between intestinal IGF1R-nulls and their wild type littermates for crypt depth and villus height respectively (Figure 2B).

Rates of enterocyte proliferation in IGF2 and IGF1R intestinal knockout mice

Rates of proliferation in both IGF2-KO and intestinal IGF1R-IKO mice were similar and statistically not different following SBR. IGF2-KO had an average proliferative index of 0.49 ± 0.02 and C57BL/6 had an average of 0.45 ± 0.01 (Figure 3A). Similarly, IGF1R-KO mice had an average proliferative index of 0.49 ± 0.01 compared to their wild type littermates with an average of 0.46 ± 0.03 (Figure 3B).

Rates of apoptosis in IGF2 and IGF1R enterocyte knockout mice

The apoptosis index in both IGF2-KO and IGF1R-IKO's were not different after SBR when compared with WT mice. IGF2-KO had an average apoptotic index of 0.025 ± 0.02 versus 0.016 ± 0.01 in WT controls (Figure 4A). IGF1R-IKO mice had an apoptotic index of 0.04 ± 0.008 compared with 0.03 ± 0.008 in WT littermates ($p=0.34$; Figure 4B).

DISCUSSION

IGF1 and IGF2 are known intestinotrophic factors [16]. Exogenous infusion of IGF1 has been shown to increase intestinal mucosal growth and enhance adaptation after SBR [4–8]. Furthermore, IGF1 is a key mediator for glucagon-like-peptide-2 (GLP-2), another known intestinotrophic factor [17]. However, IGF2 has not been previously studied in the same context despite being considered to be a significant IGF in human adults [18]. Both IGF1 and IGF2 ligands bind to IGF1R [16, 19, 20]. This background therefore provides the rationale for testing the contribution of the IGF axis toward resection-associated adaptation.

In this study, we selectively disrupted IGF2 globally and IGF1R selectively within the intestinal epithelium to determine the impact of this ligand and receptor on adaptation. We confirmed the enterocyte deletion of IGF2 and IGF1R in the knockout mice and compared the effects of SBR with their respective littermates. We found that both IGF2-KO and IGF1R-IKO mice were able to adapt normally after SBR. There were also no differences in percentage weight loss between IGF2-KO and IGF1R-IKO and their respective wild-type controls after SBR. Furthermore, we observed that the rates of proliferation and apoptosis index were also equivalent.

Our finding of normal adaptation after disrupting IGF2 and IGF1R expression would suggest that the IGF system is dispensable for normal adaptation responses to massive SBR. This would be supported by the lack of significant phenotype in the unoperated intestine of the two knockout strains. On the other hand, it must be considered that the expressions of other (non-IGF) intestinotrophic growth factors are able to compensate for a lack of IGF2 or IGF1R signaling. Double and/or triple knockout mouse strains would be necessary to test the relevance of compensatory activity or expression of other ligands or growth factor receptors.

In the case of IGF2-knockout mice, it is possible that the IGF1R is activated by other IGF ligands [21, 22]. Although not measured in this study, increased compensatory expression of

these other factors could therefore have modulated adaptation independent of IGF2. Supportive of this concept are the findings of enhanced adaptation responses to SBR following the exogenous administration of other IGF1R ligands such as IGF1 [4–8] as well as insulin [23–25].

Our finding of normal adaptation in the mice with enterocyte-targeted deletion of IGF1R expression would suggest that neither IGF1R activity in enterocytes nor IGF1 and IGF2 ligand expression are important for resection-induced adaptation. IGF1 and IGF2 are capable of binding to the insulin receptor although with a tenfold lower affinity for IGF2 and a 50- to 100-fold lower affinity binding for IGF1[26]. Therefore, it must be considered that the IGF ligands are capable of governing intestinal adaptation via the insulin receptor.

Since our intestinal IGF1R knockout mice have normal IGF1R activity in all other cells besides enterocytes, it is plausible that the IGF system drives adaptation via non-epithelial cells within the intestine. IGF1 is known to be mitogenic to myofibroblasts [27]. As such, it is possible that intestinal mucosal growth is driven by IGF1R stimulation of subepithelial mesenchymal or immune cells. This paradigm is similar to our findings of impaired resection-induced adaptation responses under multiple conditions of global inhibition of epidermal growth factor receptor (EGFR) signaling [28] but preserved responses in mice with genetically disrupted expression of intestinal epithelial EGFR [29].

We recently found that targeted enterocyte deficiency of Rb was associated with significant intestinal epithelial growth and enhanced expression of epithelial IGF2 [12]. In these mice, the trophic effects of Rb-deficiency were completely reversed by breeding with the same mutant strains (IGF2-KO and IGF1R-IKO) as in the current study. These findings would suggest that the hyperplastic mucosal phenotype associated with Rb deficiency and SBR may occur via different mechanisms. More importantly, these findings underscore the consideration that mucosal surface area expansion can be accomplished by multiple disparate, perhaps overlapping and/or additive signaling pathways. Since this is an important physiologic response, precise molecular dissection of the most important and potent intestinotrophic signaling pathways and most relevant cell compartments will be required to optimize therapeutic interventions in patients who have suffered a massive intestinal loss.

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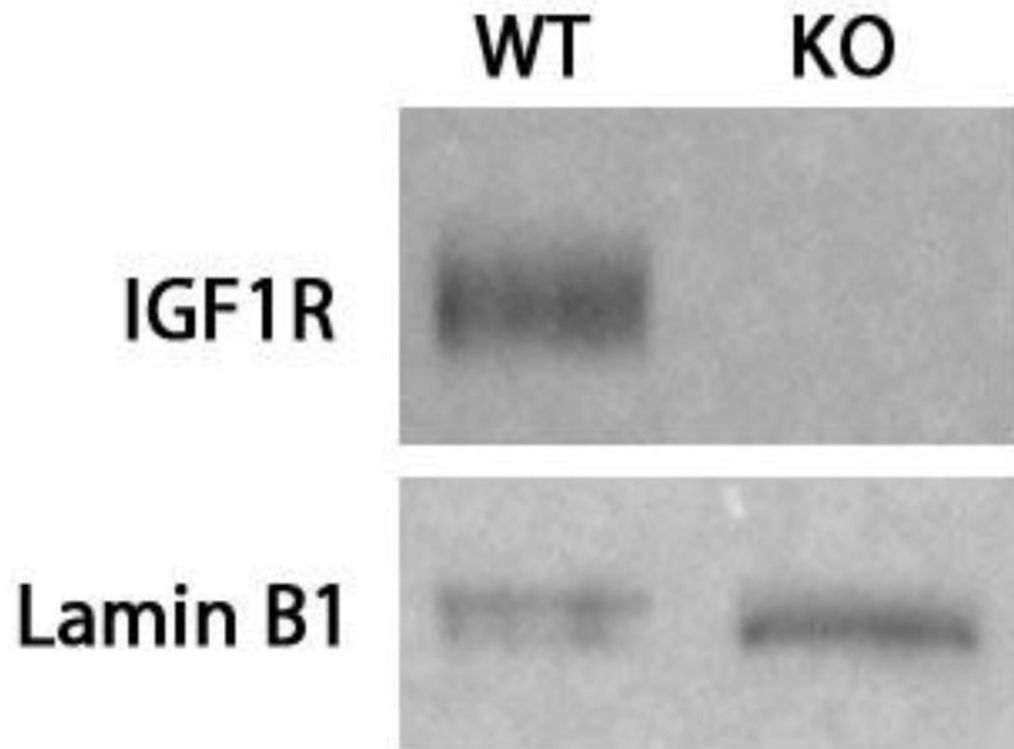


Figure 1. Western blotting confirming disrupted IGF1R expression in the crypt enterocytes. Villin Cre-ER (+); IGF1R (f/f) mice and their wild type littermates (Villin Cre-ER- (-)); IGF1R(f/f) were both injected with Tamoxifen for three days prior to resection. Lamin B1 was used as loading control. Successful deletion of IGF1R intestinal knockout was confirmed with all Villin Cre-ER(+); IGF1R (f/f) mice.

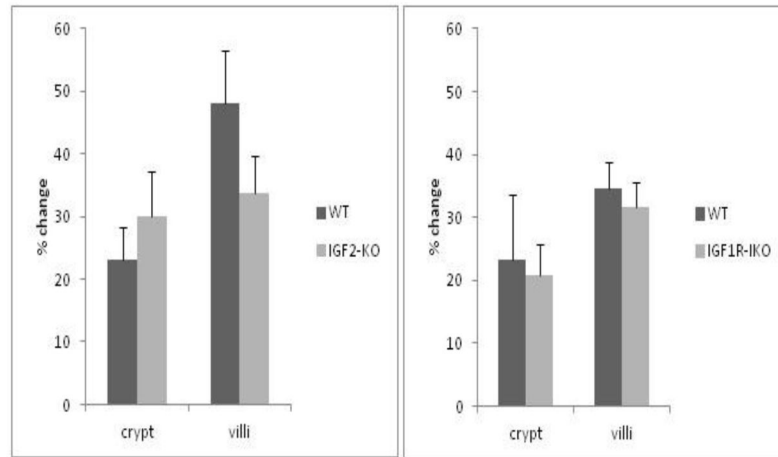


Figure 2. Percentage change in crypt depth and villus height for (A) IGF2-KO and (B) IGF1R-intestinal knockout (IKO) mice after small bowel resection. No differences in postoperative villus or crypt growth were observed between the mutant strains or corresponding wild-type (WT) mice in any parameter.

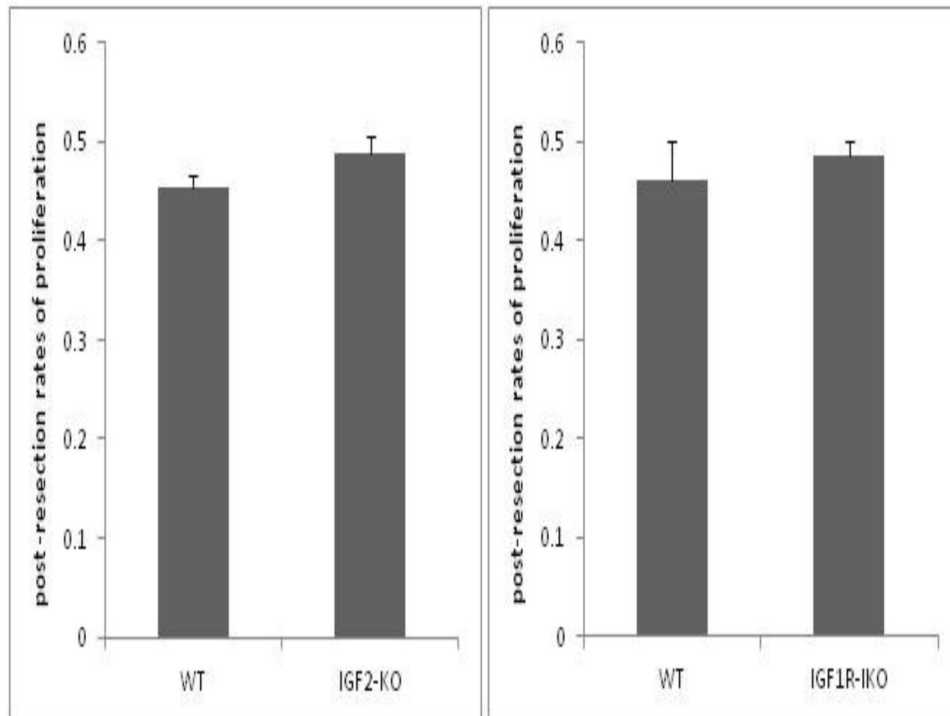


Figure 3.

Post-operative rates of crypt cell proliferation in (A) IGF2-KO and (B) IGF1R- intestinal knockout (IKO) compared to their wild type (WT) littermates. BrdU was injected 90 minutes prior to harvest. Immunostaining was performed on tissue sections and BrdU-positive stained cells were counted in each crypt. A ratio was calculated as number of cells stained positive divided by total number of cells in the crypts. A minimum of 20 crypts were counted per mouse. No statistical differences were observed between either mouse strains versus their corresponding WT controls.

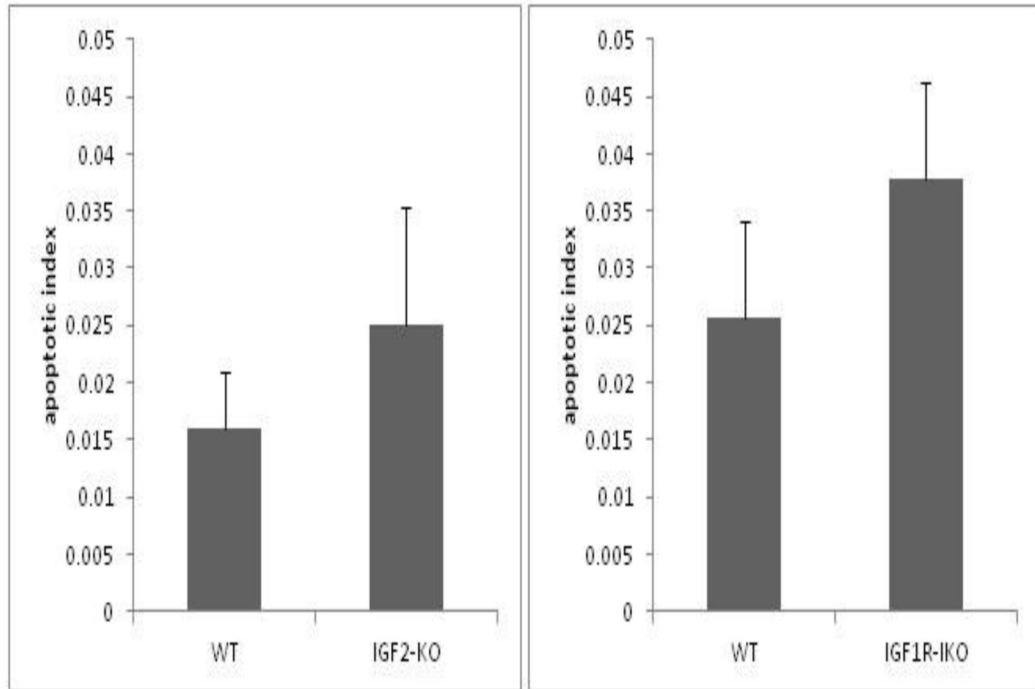


Figure 4. Post-operative crypt apoptosis index for (A) IGF2-KO and (B) IGF1R- intestinal knockout (IKO) mice compared to their wild type littermates. H&E stained sections of ileum were analyzed for apoptotic bodies. Apoptotic index was calculated as number of apoptotic bodies found per number of crypts. A minimum of 50 crypts were counted per mouse. There were no statistical differences observed between the mutant mice and their corresponding WT strains.