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Both epidermal growth factor and insulin-like growth factor receptors are dispensable for structural intestinal adaptation☆

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ABSTRACT

Purpose: Intestinal adaptation structurally represents increases in crypt depth and villus height in response to small bowel resection (SBR). Previously, we found that neither epidermal growth factor receptor (EGFR) nor insulin-like growth factor 1 receptor (IGF1R) function was individually required for normal adaptation. In this study, we sought to determine the effect of disrupting *both* EGFR and IGF1R expression on resection-induced adaptation.
Methods: Intestinal-specific EGFR and IGF1R double knockout mice (EGFR/IGF1R-IKO) (n = 6) and wild-type (WT) control mice (n = 7) underwent 50% proximal SBR. On postoperative day (POD) 7, structural adaptation was scored by measuring crypt depth and villus height. Rates of crypt cell proliferation, apoptosis, and submucosal capillary density were also compared.
Results: After 50% SBR, normal adaptation occurred in both WT and EGFR/IGF1R-IKO. Rates of proliferation and apoptosis were no different between the two groups. The angiogenic response was less in the EGFR/IGF1R-IKO compared to WT mice.
Conclusion: Disrupted expression of EGFR and IGF1R in the intestinal epithelial cells does not affect resection-induced structural adaptation but attenuates angiogenesis after SBR. These findings suggest that villus growth is driven by receptors and pathways that occur outside the epithelial cell component, while angiogenic responses may be influenced by epithelial-endothelial crosstalk.

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Intestinal adaptation is defined by structural increases in crypt depth and villus height resulting in increased mucosal surface area in response to massive small bowel resection (SBR) [1]. Epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1) [2,3] and 2 (IGF2) [4] are recognized intestinotrophic factors which bind and signal through their respective receptors, epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGF1R). Infusion of EGF [5,6] and IGF1 [2,4,7–10] are both associated with an exaggerated adaptation response to SBR.

Previously, we have demonstrated that resection induced adaptation was attenuated when the EGFR was inhibited in mutant (waved-2) mice with globally perturbed EGFR activity [11,12]. However, more recently, with the use of intestinal specific EGFR [13] and IGF1R [14] –null mice, we have found that normal adaptation occurred after SBR. These findings suggest that EGFR and IGF1R receptor activity in the stromal (non-epithelial) compartments of the small bowel may be more important for structural adaptation. Alternatively, one major receptor may be capable of fully compensating for the other in the process of regulating adaptive intestinal growth. Furthermore, enterocyte receptor signaling is likely relevant since crypt cell proliferation is significantly elevated after SBR. The purpose of this study was to directly test the hypothesis that both EGFR and IGF1R signaling in the epithelium are required for normal adaptation responses to SBR.

1. Materials and methods

1.1. Animals and experimental design

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

Intestinal epithelial-specific EGFR and IGF1R double knockout mice (EGFR/IGF1R-IKO) (n = 6) were generated using a tamoxifen inducible Villin-Cre (VC-ER (+)) recombinant system [15] which disrupts genes between tagged (floxed; (f/f)) regions. Wild type littermates VC-ER (−); EGFR (f/f); IGF1R (f/f) (n = 7) were used as control mice. Both VC-ER (+) and VC-ER (−); EGFR (f/f); IGF1R (f/f) mice received intraperitoneal injections of tamoxifen (0.5 mg/day; Sigma, St. Louis, MO) for 3 consecutive days prior to SBR. Mice were kept in the animal holding area with a 12 hour light-dark schedule and given rodent chow ad lib after weaning.

EGFR-IKO/IGF1R-IKO mice (n = 7) and their wild type (WT) littermates (n = 6) underwent 50% proximal SBR between 8 to 10 weeks

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of age. All mice were fed standard rodent liquid diet (Micro-Stabilized Rodent Liquid Diet LD 101; Purina Mills, St Louis, MO) 1 day prior to SBR. Post-operatively, mice were given water ad lib and resumed rodent liquid diet on post-operative day (POD) 1. All mice were weighed before resection and at the time of harvest on POD 7.

Ileal tissue was collected at the time of SBR as a baseline control. On POD 7, the remnant ileum was removed and histology was collected to compare adaptive structural changes within the crypts and villi with preoperative baseline measurements. Enterocytes were isolated and used for protein analysis to confirm gene deletion and to analyze downstream markers of receptor-mediated phosphorylation pathways. Rates of crypt cell proliferation, apoptosis, and submucosal capillary density were also recorded.

1.2. Small bowel resection

EGFR/IGF1R-IKO and their WT littermates underwent a 50% proximal SBR as previously described [1]. The intestinal resections were performed by transecting the bowel 1 to 2 cm distal from the ligament of Treitz and at 12 cm proximal to the ileocecal junction followed by removal of the intervening segment. Intestinal continuity was reestablished by an end-to-end primary anastomosis using interrupted 9-0 monofilament sutures. A 2 cm distal segment of the resected ileum was fixed in 10% neutral-buffered formalin for baseline histology.

1.3. Tissue isolation

All mice were sacrificed on POD 7 as previously described [13]. 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 distal to the anastomosis was fixed in 10% neutral-buffered formalin to compare with intraoperative histology. The remainder of the distal segment of bowel was used to isolate crypt and villus enterocyte using our laboratory published protocol [16]. Protein from enterocytes was used to verify the efficiency of deletion of EGFR and IGF1R.

1.4. Histology

Intestinal tissue was cut into two sections each, 50 μm apart followed by H&E staining. Crypt depth and villus height were measured using MetaMorph computer program (Molecular Devices, Dowington, PA). At least 20 villi and crypts were measured per mouse. Post-operative and intra-operative crypt depth and villus height were compared to calculate the magnitude of the structural adaptation.

1.5. Cell proliferation and apoptosis

Paraffin-embedded tissue sections were immunohistochemically stained with p-histone 3 as previously described [17]. Rates of proliferation were determined by counting the number of cells staining positive with p-histone 3 divided by the total number of cells in each crypt. A minimum of 20 crypts were counted per mouse.

H&E stained slides were analyzed for apoptotic bodies (pyknotic nuclei, condensed chromatin, and nuclear fragmentation) [18]. An apoptosis index was determined by counting the number apoptotic bodies found within 50 well-oriented crypts.

1.6. Submucosal capillary density

Small intestine tissues were immunohistochemically stained with cd31 to determine the capillary density in the submucosal layer. Immunohistochemistry staining with cd31 was performed using our previously published protocol [19]. The number of cd31-stained vessels was counted in the submucosal layer per high power field (40x). Ten well oriented power fields per mouse were counted and averaged.

1.7. Western blotting

Crypts were isolated and then lysed with sodium dodecyl sulfate (SDS) lysing buffer (50 mM Trist-HCL pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 5% mercaptoethanol). The cell samples were sonicated and heated in 100 °C for 5 minutes. Protein quantification was performed using a RC DC kit (Bio-Rad, Hercules, CA). Equal amounts of protein were separated in 10% SDS-PAGE gel and probed with their respective antibodies. (EGFR, IGF1R, p-ERK(Thr202/Tyr204), p-AKT(Ser473), Actin (Cell Signaling Technology, Danvers, MA); Tubulin (EMD Millipore, Darmstadt, Germany)).

1.8. Statistical analysis

All values are reported as mean ± standard error of the mean. Statistical analysis was performed using Student's t-test to compare the two experimental groups. A p-value of less than 0.05 was considered significant.

2. Results

2.1. EGFR/IGF1R-IKO mice demonstrate normal structural adaptation after SBR

The successful deletion of EGFR and IGF1R protein within enterocytes of the small intestine was confirmed by Western blot (Fig. 1a). Both intestine-specific single EGFR (EGFR-IKO) and IGF1R knockout mice (IGF1R-IKO) have been previously characterized and

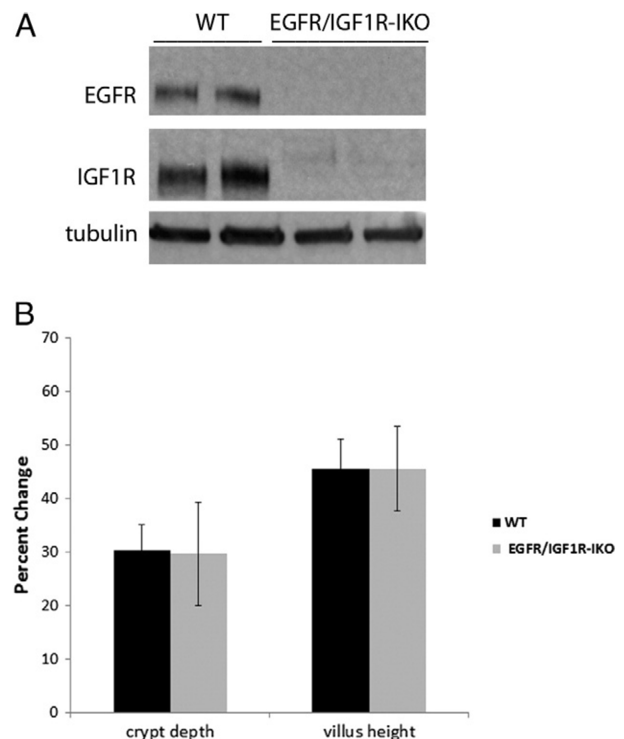

Fig. 1. A) Western blot confirming deletion of EGFR and IGF1R protein expression in crypt enterocytes. Both EGFR and IGF1R expression were knocked out in the intestinal epithelium in adult mice (EGFR/IGF1R-IKO; n = 6) following the injection of tamoxifen. Both the knockout mice and wild-type (n = 7) controls were administered tamoxifen for three days prior to resection. Tubulin was used as loading control. Successful deletion of EGFR and IGF1R protein was confirmed with all Villin Cre-ER(+); EGFR (f/f),IGF1R (f/f) mice. B) Percentage increase in crypt depth and villus height for EGFR/IGF1R-IKO (n = 6) and WT (n = 7) mice after small bowel resection. There were no statistical differences in post-operative villus or crypt growth between groups.

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