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Changes in Morphology and Function in Small Intestinal Mucosa after Roux-en-Y Surgery in a Rat Model

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Abstract

Background—Currently there is no an appropriate model to study intestinal mucosal atrophy in vivo that preserves the nutritional status of the organism.

Materials and Methods—We created a defunctionalized segment of jejunum via a dead-end Roux-en-Y anastomosis in rats. We compared tissue morphometric parameters in the intestinal mucosa of the defunctionalized bowel to that of the mucosa proximal and distal to the anastomosis. We further measured ERK activation within the mucosa as well as sucrase-isomaltase and DPPIV levels as markers of intestinal mucosal differentiation by Western blotting of mucosal scrapings.

Results—Three days after anastomosis, the defunctionalized bowel exhibited decreased diameter and thickness of both the mucosa and the fibromuscular layer compared with adjacent bowel in continuity for luminal nutrient flow or with bowel from control animals. Sucrase-isomaltase and DPPIV levels were also decreased. Furthermore, mucosal ERK activation, assessed as the ratio of phosphorylated to total ERK, was also reduced. Animal weights did not differ between bypassed and control animals.

Conclusions—Deprivation of nutrient flow in a segment of bowel by defunctionalizing Roux-en-Y anastomosis produces mucosal atrophy as indicated by altered histology, differentiation marker expression, and ERK signaling, in animals that are otherwise able to maintain enteral nutrition.

Keywords

Atrophy; ERK; Differentiation; Sucrase-isomaltase; DPPIV

Introduction

The intestinal mucosa atrophies during fasting and in patients with ileus or sepsis who are not eating. Total parenteral nutrition (TPN) of laboratory animals has been used as a model to study mucosal atrophic processes (1, 2). Although TPN induces intestinal atrophy (3), it also has profound effects on intraepithelial cytokine signaling (4) and mucosal immune defense (5, 6) that may be independent of the mucosal atrophy itself. TPN also promotes ion transfer and permeability across the intestine in experimental animals (7, 8) that may change the pharmacokinetics or effects of drugs being tested for use in patients with mucosal intestinal pathology.

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In addition to growth factors and intraluminal nutrients, repetitive mechanical deformation engendered by peristalsis or villous motility also modulates intestinal epithelial biology (9-11). In vitro, repetitive deformation stimulates the proliferation of intestinal epithelial cells on collagen substrates, modulates differentiation marker expression, and activates p38 and JNK signaling in intestinal Caco-2 cells in response to repetitive deformation (12). In vivo, repetitive deformation alone stimulates intracellular signaling within small intestinal or colonic mucosa in anesthetized rats (13). Suppressed contractility and diminished luminal nutrient flow reduce metabolism and initiate mucosal atrophy (1). In vitro, Extracellular signal-Regulated Kinases (ERK) signaling critically mediates the effects of repetitive deformation on human Caco-2 intestinal epithelial cells (14). However, the signaling mechanisms involved in mucosal atrophy have not been characterized in vivo. We hypothesized that creating a blind Roux en-Y anastomosis in rat jejunum will lead to mucosal atrophy and reduction of function in the defunctionalized intestinal segment which is deprived of luminal flow, while still permitting normal enteral nutrition and preserving normal biology in other segments of the intestine in the same animal.

Methods

Animals and surgical procedures information

All experiments were approved by the University Laboratory Animal Resources at Michigan State University. 3 month old female Wistar rats were purchased from Charles River (Wilmington, MA) and were housed using a 12 hour light/12 hour dark cycle. Diets and water were provided *ad libitum*. Control animals were maintained on regular diet until one day before harvest. Sham and anastomosis operated rats were kept on liquid diets for two days before surgery and sacrificed three days postoperatively. After surgery, each animal was maintained on liquid diet for three days. An additional group of animals was kept for 30 days after defunctionalizing anastomosis and samples were then analyzed morphologically. Sham-operated animals served as controls.

Roux-en-Y anastomosis—To create a defunctionalized segment of jejunum in continuity with the remainder of the bowel, we performed a defunctionalizing Roux-en-Y anastomosis in rats through a midline laparotomy. The jejunum was divided 1 cm from the ligament of Treitz using 5-0 silk sutures (Ethicon, Inc., Somerville, NJ). The proximal jejunum (proximal limb) was anastomosed to the distal jejunum (distal limb) three cm more distally than the original transection with a side to side anastomosis using running 7-0 vicryl sutures (Figure 1A-B). In control operated animals the abdominal wall was opened and the intestine was manipulated and measured. In sham-operated animals, the small intestine was transected twice in the jejunum at sites 4 centimeters apart, at sites equivalent to those in which the experimental defunctionalizing Roux-en-Y anastomosis transections were performed. However the bowel was then reconstructed in continuity with two end to end anastomoses constructed with 7-0 vicryl suture. The abdominal cavity was closed using a running 5-0 vicryl suture. The skin was closed by using surgical staples.

Morphometric analysis

Samples of the intestinal segments from the proximal, distal and Roux limbs were fixed in 10 % formalin for 24 hours and embedded in paraffin. Step sections (4.0 m thick) were prepared from all of the blocks and stained with hematoxylin and eosin (H&E). Sections were visualized and photographed on a Nikon Microphot-FXA (Nikon Microphot-FXA). Villous height and thickness of the fibromuscular layer was measured on transverse sections stained with hematoxylin and eosin using ImageJ software (NIH, Bethesda, MD) each compared with bowel proximal and distal to the defunctionalized segment from the same animal and with specimens from sham-operated animals at the same sites.

Protein isolation and Western blotting analysis—Mucosal scrapings from target intestinal segments after sacrifice and harvest were immediately immersed in ice cold lysis buffer. Tissue was homogenized using BulletBlender (Next Advance, Averill Park, NY) and then centrifuged at 15,000g for 10 min at 4°C and resolved by SDS-PAGE as previously described (14). Membranes were blotted for sucrase-isomaltase, Dipeptidyl peptidase-4 (DPPIV), Phospho-ERK Thr 202, Tyr204 or total ERK with appropriate antibody (Cell Signaling Technology, Danvers, MA) as previously described (15). Membranes were then re-probed with antibodies specific for α -Actin and appropriate fluorophore conjugated secondary antibody as a loading control. Bands were visualized using Odyssey imaging system (Licor, Lincoln, NE) and analyzed with the Kodak Image Station 440CF. All exposures of probed membranes used for densitometric analysis were within the linear range.

Data analysis—Values are reported as group means $\pm$ standard error of the mean (SEM) of the non-transformed data. The differences between control and experimental groups were compared using paired or unpaired t-tests as appropriate. Prior to analysis, all data were checked to ensure they fit a normal distribution. Orthogonal contrast comparisons for the effect of surgical procedures on sucrase-isomaltase, DPPIV and ERK signaling were conducted (Control vs. Sham and Anastomosis; Sham vs. Anastomosis) as previously described (16). Differences between means were considered significant at $p < 0.05$.

Results

Body weight and intestine at harvest

Body weight did not differ in experimental animals at three days after surgery compared to sham-operated controls (208.3 ± 2.7 g vs. 213.5 ± 8.7 g). Three days after the surgery, the proximal and distal limbs contained chyme, the anastomosis was patent (Figure 1A-B), and the defunctionalized limb was empty.

Loss of function changes histomorphometry of small intestine—Three days after surgery, the diameter of the defunctionalized intestine was significantly reduced compared to either the control, sham-operated controls or to the bowel previously immediately proximal or distal to the segment that had been defunctionalized and now immediately proximal or distal to the anastomosis ($p < 0.05$, Figure 1B-C). Hematoxylin-eosin-staining confirmed parallel alterations in histology. The thickness of the mucosal layer was significantly decreased ($p < 0.05$) primarily due to villous shortening, whereas the thickness of stromal layer was not changed (Figure 2A-B). Total thickness of intestinal wall of defunctionalized limb was significantly reduced compared to control ($p < 0.05$, Figure 1C). The ratio of the thickness of the intestinal mucosa to the thickness of the fibromuscular layer did not differ among defunctionalized, proximal, and distal jejunum. In rats maintained for 30 days after defunctionalizing anastomosis, the defunctionalized bowel diameter was decreased $34.2 \pm 8.39\%$ and the mucosal villous height reduced by $34.7 \pm 4.7\%$ compared to the adjacent proximal bowel ($p < 0.05$ for each, data not shown). In contrast, thickness of the fibromuscular layer in the defunctionalized gut increased $15.65 \pm 1.2\%$ at 30 days compared to the proximal bowel. ($p < 0.05$ for each, data not shown).

Defunctionalization is associated with reduced brush border enzyme levels—

The level of DPPIV protein was $65.2\% \pm 7.9\%$ and $59.2\% \pm 9.4\%$, lower in the defunctionalized intestinal mucosa than in the mucosa of the proximal or distal bowel respectively ($n=6$, $p < 0.05$, Figure 3A). Similarly sucrose-isomaltase protein was $61.7\% \pm 8.8\%$ and $63.8\% \pm 8.3\%$ lower in the defunctionalized intestinal mucosa than in the mucosa of the proximal or distal bowel respectively ($n=6$, $p < 0.05$, Figure 3B). Sucrase-isomaltase

and DPPIV were also lower in the defunctionalized mucosa than in the same segment of bowel from control or sham-operated animals ($n=3-6$, $p<0.05$, Figure 3A-B). Levels of DPPIV and sucrase-isomaltase were not different among segments of intestine in sham operated animals. The intake of the liquid diet for five days as well as surgical procedures reduced average level of sucrase-isomaltase in sham and anastomosis operated group ($p<0.05$, figure 3C).

Defunctionalization is associated with reduced intracellular signaling

Levels of phosphorylated and total ERK was detected by western blot and compared to α -actin levels. ERK activation (pERK/ERK relative to α -actin) was reduced by $61. \pm 10.1\%$ and $46.7 \pm 13.9\%$ at 3 days vs. proximal or distal gut ($p<0.05$, Figure 4).

Discussion

Intestinal mucosal atrophy is a common problem in patients undergoing prolonged periods of fasting during ileus, sepsis, or starvation. The flow of luminal contents modulates intestinal mucosal structure and enterocyte function (1, 17). In the absence of luminal nutrients, mucosal epithelial cells lose their function, reduce their absorptive capacity, and slow their proliferation (7, 18, 19). Changes in cell and tissue architecture and loss of intestinal epithelial integrity lead to an impaired histostucture, increased permeability, and failure of the intestinal mucosal barrier.

In this work, we present an experimental model that allows the study of intestinal mucosal atrophy without the neurohumoral changes or technical difficulties that accompany total parenteral nutrition. We generated a defunctionalized segment of the jejunum lacking nutrient flow but still preserved the ability of the animal to be nourished enterally. Loss of function due to reduced interaction of luminal contents with mucosa initiates morphological and biochemical atrophy of the intestinal mucosa that correlates with decreased ERK signaling. In a previous preliminary study, we attempted to study the impact of defunctionalization on mucosal healing after ulcer formation in mice undergoing Roux-en-Y anastomoses (20) but these observations were confounded by the smaller diameter of the mouse intestine that led to partial obstruction and dilation of the defunctionalized intestinal segment not observed here in the slightly larger rat model.

The ability of deprivation of contact with luminal nutrients to induce atrophy is consistent with previous observations that the intestinal mucosa changes morphologically in stimuli. Intestinal villi increase height during intensive nutrient absorption during lactation (21) or after small intestine resection (22). Conversely, the mucosa atrophies in patients subjected to starvation, exhibiting reduced villous height and crypt length and impaired intestinal permeability (23). Prolonged loss of function in the intestine over long period of time was associated with even more pronounced morphologic changes 30 days after surgery. Similarly, in rats villous height in jejunum is reduced after 8 days on TPN (1). The defunctionalization model may be of interest to laboratories that do not have access or resources for rodent TPN or that wish to avoid the possible systemic confounding effects of TPN such as increased permeability (8) (less likely to be a problem in our model because only a small segment of the gut exhibits mucosal atrophy) and systemic defects of cell defense (24). In addition, the defunctionalizing anastomosis model permits direct paired comparisons of mucosal biology in atrophic and non-atrophied mucosa in the same animal in adjacent segments of bowel, which may be experimentally useful for some purposes.

Why small intestinal mucosal atrophy occurs in fasting or starving patients is an interesting and poorly understood question. Although the small bowel mucosa generally derives most nutrition from luminal rather than circulatory nutrients in normal function, total parenteral

nutrition should be able to provide the most relevant nutrients to the mucosa by perfusion in a manner similar to that by which other organs are perfused. The hypothesis that mucosal atrophy reflected a relative glutamine deficiency not treated by conventional total parenteral nutrition solutions has not been substantiated in human studies (17). Our data clearly demonstrates that the mucosal atrophy seen with TPN is not a direct consequence of TPN itself or of a neurohumoral response to an overall absence of nutrients within the entire bowel. There may be something special about the local presence of luminal nutrients, either based upon apical vs. basal cell sensors or the existence of concentration gradients across the mucosa that is sensed by the gut epithelium.

We have previously demonstrated that physical forces like repetitive deformation (9) or pressure (25, 26) can stimulate intestinal epithelial proliferation and regulate intestinal epithelial differentiation in vitro. Interestingly, both the mitogenic and differentiating properties of repetitive deformation appear independent of the effects of glutamine (27). Luminal nutrients stimulate both peristalsis (28) and villous motility (29). Moreover, as the luminal contents are relatively non-compressible, passage of luminal contents through the bowel deforms the villi and the mucosa by a combination of pressure and shear stress. (30). Increases in extracellular pressure can also stimulate intestinal epithelial proliferation (20). It therefore becomes attractive to hypothesize that at least one of the factors contributing to mucosal atrophy in the present model is alterations in the physical forces associated with luminal nutrition that repetitively deform the mucosa. Such forces similarly stimulate cell proliferation in the cardiovascular system (31), the lung (32, 33) and other tissues (34). Additionally in this model of intestinal atrophy in defunctionalized limb atrophic processes are limited to a small section of intestine, thus one would not expect to have systemic effects from increased bacterial translocation due to increased intestinal wall permeability as is observed in the TPN model (8). Our model differs from and may be more extreme than pure human starvation in that starvation may nevertheless have gastric, biliary and pancreatic secretions flowing through the lumen of the gut. However, in critically ill fasting patients in the hospital with nasogastric tubes gastric secretions and some biliary and pancreatic secretions are diverted proximally out the tube and less pass through the bowel lumen, so this scenario may more closely resemble our current model.

The expression level and activity of brush border enzymes and intestinal epithelium-specific functional proteins are conventionally used to assess the differentiation of intestinal epithelial cells (35). The brush-border enzyme DPPIV is amplitude-dependently activated by repetitive strain in human Caco-2 intestinal epithelial cells (11) or murine enterocytes (36). DPPIV was reduced during azathioprine-induced gastroenteritis and small bowel villus atrophy (37). Also DPPIV activity decrease in intestine of starved for three days rats (38) but not in rats after TPN (39). Sucrase-isomaltase is also expressed in differentiated enterocytes, and its level is reduced in the atrophic intestinal mucosa of methotrexate-treated rats (40). Here, we observed that defunctionalization of a jejunal segment reduced levels of these differentiation markers within three days after surgery. Our results suggest that jejunal sucrase-isomaltase may be more sensitive to the change of diet and jejunal transection than DPPIV levels or ERK signaling, independently of defunctionalization. This reduction in brush border enzymes appears to correlate with histological evidence of atrophy in the defunctionalized rat intestinal mucosa.

ERK activation is a potent mitogenic stimulus that can also induce intestinal epithelial differentiation, activated by a variety of growth factors (41) and cytokines (42). Repetitive deformation also stimulates proliferation and regulates enterocytic differentiation via a complex signal pathway (10) that involves tyrosine kinase and PKC activation and downstream ERK-mediated signaling (12). These observations are consistent with our present observation that ERK signaling is decreased in the atrophic defunctionalized mucosa

in vivo. The extent to which this decrease in ERK activity actually influences the biochemical and morphological mucosal atrophy we observed awaits further study beyond the scope of this report.

In summary, the absence of interaction with luminal chyme changes intestinal morphology in a defunctionalized loop of rat intestine. Morphologic evidence of mucosal atrophy correlated with a reduction in mucosal differentiation and ERK signaling. Formation of a defunctionalizing Roux-en-Y anastomosis may be an appropriate model to investigate mucosal atrophy and the effects of luminal chyme interactions with the bowel in intact otherwise enterally-fed rodents.

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Figure 1. Defunctionalized intestine changes morphology within three days after surgery

A. General view of the small intestine of rat after creation of defunctionalized limb in small intestine of rat. Jejunum was divided and was transposed to the distal part of the jejunum approximately three cm from the transaction point, where it was anastomosed in a Roux-en-Y end to site fashion, thereby creating a defunctionalized section of jejunum, B. General view of intestine at the time of harvest. Dissected intestinal segments three days after anastomosis surgery. C. Diameters of intestinal segments of defunctionalized (defunct) Roux-en-Y anastomosis vs sham operated animals (control) (n=8, p<0.05). * - significantly different from proximal and distal limb of same group, # - significantly different from area representing defunctionalized region of intestine in Control or Sham operated animals.

Figure 2. Histological parameters of small intestine of Roux-en-Y anastomosis three days after surgery

A. Mucosal thickness of defunctionalized (defunct) Roux-en-Y intestinal wall anastomosis vs sham-operated animals (control) (n=6-8, $p<0.05$). B. Stromal thickness of Roux-en-Y intestinal wall anastomosis vs sham operated animals (control). (n=6-8, $p<0.05$). C. Total thickness of Roux-en-Y intestinal wall anastomosis vs sham operated animals (control). (n=6-8, $p<0.05$). * - significantly different from proximal and distal limb of same group, # - significantly different from area representing defunctionalized region of intestine in Control or Sham operated animals.

Figure 3. Level of brush border enzymes is reduced in defunctionalized intestine compared with proximal or distal limbs or sham operated animals

Levels of DPPIV (A) or sucrase-isomaltase (SI) (B) were lower in the defunctionalized (defunct) intestinal mucosa three days after anastomosis compared to sham-operated controls or proximal (prox) or distal bowel in the same animals ($p < 0.05$, $n = 3-7$). * - significantly different from proximal and distal limb of same group, # - significantly different from area representing defunctionalized region of intestine in Control or Sham operated animals, **,- significantly different comparison of Control vs Sham and Anastomosis groups ($p < 0.05$).

Figure 4. ERK signaling changes in defunctionalized intestine comparing with proximal or distal limbs or sham-operated animals

The proportion of phosphorylated ERK was significantly reduced in defunctionalized (Def.) limb of intestine on the third day after anastomosis compared to sham-operated controls or proximal (Prox.) or distal bowel in the same animals ($p < 0.05$, $n = 4-7$). * - significantly different from proximal and distal limb of same group, # - significantly different from area representing defunctionalized region of intestine in Control or Sham operated animals.