Time-dependent intestinal adaptation and GLP-2 alterations after small bowel resection in rats

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RESECTION OF THE SMALL BOWEL is a powerful stimulus to morphological and functional adaptation of the remaining intestine, and this response has been intensively studied in animal models. The diameter of the remaining small bowel is increased, as are villus length, crypt depth, and muscle mass (6, 12, 30). All layers of the bowel wall are believed to participate in the adaptive response, which primarily develop over 2 wk and is stated to be fully established by 1 mo in the rat (6, 12). Adaptation takes place on either side of the resected small bowel, but the ileum is believed to have a greater capacity to adapt than the jejunum (35, 37). The colon also demonstrates some degree of adaptive growth after small bowel resection, but the magnitude and duration seem closely related to the extent of resection (28, 38, 39). However, the morphological in-

formation derived from these cited studies was obtained from nonrandomly selected fields of vision and has not been supported by efficient and unbiased morphological estimates.

Glucagon-like peptide-2 (GLP-2) is a 33-amino acid peptide encoded carboxy terminally to glucagon-like peptide-1 (GLP-1) in proglucagon. GLP-2 is cosecreted with GLP-1, oxyntomodulin, and glicentin from the enteroendocrine L cells, which are mainly located in the ileum and the proximal colon. It has recently been shown that GLP-2 has a highly tissue-specific trophic effect on the small intestine (7, 33) and is able to augment the adaptive response to intestinal resection in the rat (29), and plasma levels of GLP-2 have been correlated with the intestinal hyperplasia seen in streptozotocin-induced diabetic rats (9, 32). The role of endogenous GLP-2 in the adaptive intestinal response after resection has not been established yet.

The purpose of this study was to provide new and firm estimates of the time-dependent adaptive changes in the intestinal wall and surface area of the small and large bowel after small bowel resection and thus establish whether all layers of the bowel wall participate, whether there are regional differences, and to what extent the proximal colon participates in the adaptive response. Finally, this study aimed at correlating the adaptive response with plasma levels of the intestinotrophic candidate GLP-2.

MATERIALS AND METHODS

Animals

One hundred female Wistar rats (Møllegården Breeding Centre, Ejby, Denmark) weighing 174–227 g were used. They were housed individually and acclimatized for 1 wk before surgery. Animal quarters were lit in alternate 12-h cycles. All procedures were carried out in accordance with the Danish legislation regarding the care and use of laboratory animals.

Design

Rats were randomized to either resection or sham operation and were observed for 0, 4, 7, 14, and 28 days.

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Surgical Procedures

Nonfasted rats were given atropine sulfate (0.05 mg/kg) and ampicillin (Anhypon, 100 mg/kg) subcutaneously and were anesthetized by subcutaneous injection of fentanyl-fluanisone (Hynopon, 0.3 ml/kg) and midazolam (Dormicum, 3.75 mg/kg) before surgery. A subcutaneous injection of 10 ml of isotonic saline was given preoperatively to prevent dehydration. After a midline laparotomy, the small bowel was marked 5 cm distal to the ligament of Treitz and 10 cm proximal to the ileocecal valve, marking out ~80% of the small bowel. The rats were then randomized to either resection or sham operation. Before transection of the small bowel at the distal marking, a nonabsorbable suture (6–0 Prolene) was placed 2 cm distally and proximally to the anastomosis to achieve standardized jejunal and ileal segments of equal length and location, which could later be compared without considering changes in length caused by the experimental settings. In addition, a 4.5-cm proximal colon segment (2 cm distal to the cecum) was marked out (Fig. 1). The resected small bowel segment was rinsed, and the weight and length were determined. Sham-operated rats were transected and reanastomosed. The abdominal wall was closed in two layers. The rats received a single dose of buprenorphine (Temgesic, 0.01 mg/kg) subcutaneously for postoperative pain treatment.

Food Intake

To secure similar food intake in the two groups, the sham-operated group was given the measured mean daily food intake per gram body weight of the resected group for a given postoperative day. All the food offered was readily consumed by the sham-operated rats.

Experimental Procedures

Stereological analyses. The determinations of luminal surface areas (2) and volume fractions (10) were based on vertical sections of the tissue. The sampling procedure for vertical sections has recently been described in detail (22), and this procedure was followed (Fig. 1).

Briefly, the sections were counted by use of an Olympus BX50 microscope with a personal computer and a monitor connected to a video color camera mounted on top of the microscope. With the use of the Computer Assisted Stereological Toolbox-Grid software (Olympus), the stereological probes (points and cycloids) were superimposed onto the video images of the tissue sections viewed on the monitor (Figs. 2 and 3). Fields of vision were systematically randomly sampled in the sections by using a fixed step length (1,600 or 800 μm) accomplished by a motorized specimen stage controlled by the program. The estimates of the weights of the different intestinal layers are based on the assumption that the specific gravity is equal and ~1.0 in all layers of the bowel wall. All stereological counting procedures were performed in a blinded fashion to prevent observer bias.

The growth rates in the jejunal and ileal segments were compared to investigate possible regional differences in the small bowel adaptive response. This was done by testing differences in the means of the ratios of corresponding jejunal and ileal layers on days 0 and 28 among resected rats, as follows

\[
\frac{x_{28} - x_0}{x_0} = \frac{y_{28} - y_0}{y_0} \Rightarrow \frac{x_0}{y_0} - \frac{x_{28}}{y_{28}}
\]

where \(x_n\) is the weight of the jejunal layer on day \(n\) and \(y_n\) is the weight of the ileal layer on day \(n\).

Blood samples and assays. All animals were killed within 3 h, from 0900 to 1200. Blood was sampled from the inferior vena cava on days 0, 4, 7, 14, and 28, a few minutes before sampling of the bowel specimens. Plasma enteroglucagon and GLP-2 concentrations were measured after extraction of plasma with 61% and 75% ethanol (vol/vol, final concentration). The enteroglucagon RIA was directed against a mid sequence of the glucagon molecule comprising residues nos.
6–15 (antibody code no. 4304) and therefore also measures glucagon-containing peptide moieties of intestinal origin (gli- centin and oxyntomodulin) (14, 15). For this assay, sensitivity was below 2 pmol/l, intra-assay coefficient of variation was below 6% at 40 pmol/l, and recovery of standard (human glucagon identical to rat glucagon), added to plasma before extraction, was corrected for losses inherent in the plasma extraction procedure. GLP-2 concentrations in ethanol-extracted plasma were measured using an RIA employing antiserum code no. 92160 and standards of human GLP-2 (proglucagon 126–158, a gift from Novo Nordisk, Bagsvaerd, Denmark) and monoiiodinated Tyr-12 GLP-2, specific activity >70 MBq/nmol (13). The antiserum is directed against the N-terminus of GLP-2 (identical for rat and human GLP-2) and therefore measures only fully processed GLP-2 of intestinal origin. Sensitivity was below 5 pmol/l, and intra-assay coefficient of variation at 60 pmol/l was 6%.

The IGF-I RIA was based on a polyclonal rabbit antiserum (Nichols Institute Diagnostics, San Juan Capistrano, CA), monoiiodatated 125I-IGF-I (Novo Nordisk) and was calibrated against rhIGF-I (Amgen Biologicals). The intra- and inter-assay coefficient of variation percents were 5 and 10%, respectively.

Statistics

As expected and clearly depicted in the figures illustrating the small bowel adaptive growth, the time-dependent growth was different in the two groups, which was confirmed by a two-way analysis of variance (P < 0.001). However, the same test applied on the colonic morphological data could not confirm a difference in the time-dependent growth. Data on GLP-2, enteroglucagon, and morphological ratios were natural log-transformed for better approximation to the normal distribution. The Student’s t-test was used to compare the groups of interest, and 95% confidence limits (CL) for differences between means were calculated. Equal variances were not assumed for the morphological data.

RESULTS

Body Weight

Changes in percentage of the preoperative weights are shown in Fig. 4. Both study groups lost weight toward day 4, whereafter they started to gain weight. There was no difference in weight gain at postoperative day 28.

Small Bowel Morphology

The most pronounced growth took place in the first postoperative week, when the growth of the jejunal and ileal mucosa was 120 (P < 0.05, CL = 0.12–0.32 g) and
115% (P < 0.05, CL = 0.32–0.49 g), respectively, and with growth occurring mainly in the epithelial and lamina propria layers of the mucosa (data not shown). The muscular layer demonstrated similar growth with 134% (P < 0.001, CL = 0.14–0.30 g) and 89% (P < 0.05, CL = 0.14–0.30 g), respectively. Correspondingly, the muscular layer demonstrated similar growth with 133% (P < 0.001, CL = 0.35–0.61 g) and 162% (P < 0.05, CL = 0.52–0.82 g), respectively. Correspondingly, the muscular layer demonstrated an increase of 223% (P < 0.05, CL = 0.096–0.21 g) and 133% (P < 0.05, CL = 0.14–0.30 g).

At the end of the study period (day 28), the jejunal and ileal mucosae were increased by 209% (P < 0.05, CL = 0.35–0.61 g) and 162% (P < 0.05, CL = 0.52–0.82 g), respectively. Correspondingly, the muscular layer demonstrated an increase of 223% (P < 0.05, CL = 0.096–0.21 g) and 133% (P < 0.05, CL = 0.14–0.30 g).

To test whether the data were compatible with a stagnation of the adaptive response during the last 2 wk of the study, the mean weights on days 14 and 28 for all of the different intestinal layers were compared. No significant differences were found for any of the morphological parameters estimated.

The test for regional differences (see Stereological analyses) showed that the jejunal-to-ileal ratio had increased significantly from day 0 to day 28 for the mucosa (P < 0.01) and muscularis propria (P < 0.01) but not for the submucosal layer, suggesting a larger relative adaptive growth of the jejunum than the ileum.

**Luminal Surface Area**

The luminal surface area had increased by 190% (P < 0.05, CL = 40–79 cm²) in the jejunal segments and by 155% (P < 0.05, CL = 68–116 cm²) in the ileal segments after 28 days (Fig. 5).

**Large Bowel Morphology**

Four days after the small bowel resection, there was an increase of 30% (P < 0.05, CL = 0.02–0.1 g) in the weight of the colonic mucosal layer (Table 1), which was further increased to 41% (P < 0.05, CL = 0.04–0.2 g) at day 7. There was no further increase in the mucosal weight at day 14, and at the end of the study period the difference between groups was no longer present. The mucosal growth was accompanied by an increase in luminal surface area of the same range and duration (Fig. 5). The growth of the submucosal and muscular layers displayed the same pattern as the mucosal growth, without reaching statistical significance.

**Enteroglucagon and Glucagon-like Peptide-2**

Enteroglucagon and GLP-2 measurements displayed an almost identical pattern of response to small bowel resection. Enteroglucagon levels were on average (data pooled from day 4 to day 28) increased by 106% (P < 0.0001, CL = 77–137%) in the resected group compared with the sham-operated group, and correspondingly GLP-2 levels were increased by 130% (P < 0.0001, CL = 89–179%) (Fig. 6).

**IGF-I**

On day 4, serum IGF-I levels were reduced by 49% (P < 0.001, CL = 178–294 µg/l) in resected rats and by 49% (P < 0.001, CL = 190–323 µg/l) in sham-operated rats compared with day 0 values (Fig. 6). Baseline values were not reached for either of the two groups within the study period.

**DISCUSSION**

The existing data on intestinal adaptation after small bowel resection are, because of the methodological problems and limitations of previous studies, potentially biased. Accordingly, the present study contains the first firm and unbiased measurement of the adaptive small bowel and colonic growth during the first 4 wk after extensive small bowel resection. There was a significant, early, and persistent adaptive growth response in the remaining small bowel, which involved the entire intestinal wall and markedly increased the luminal surface area, whereas the early growth response in the colon was of a temporary nature. In contrast to what is generally believed, we found that the adaptive growth was stronger in the jejunum than in the ileum. The continuously elevated GLP-2 level in the resected rats is consistent with the hypothesis that GLP-2 plays an important role in the adaptive response after small bowel resection.

Review articles on the subject of intestinal adaptation after intestinal resection have primarily focused on the adaptive response of the mucosa, which is probably justified from a clinical viewpoint (5, 27, 37),

| Table 1. Mean differences in percentage between resected and sham-operated rats for the mucosal, submucosal, and muscular layers |
|-------------------------------------------------------------|---------------------------------|----------------------------------|---------------------------------|
|                                      | Mucosa  | Submucosa | Muscularis Propria |
|                                      | Jejunum | Ileum     | Colon              | Jejunum | Ileum     | Colon              | Jejunum | Ileum     | Colon              |
| Day 4                                | 95%     | 81%       | 30%                | 70%     | 35%       | 24%                | 75%     | 45%       | 20%                |
|                                      | (0.17g) | (0.27g)   | (0.07g)            | (0.007g)| (0.009)   | (0.006g)           | (0.041g)| (0.058g)  | (0.03g)            |
|                                      | (0.11–0.23g) | (0.23–0.32g) | (0.02–0.1g)       | (0.006–0.01g) | (0.003–0.02g) | (–0.002–0.02g) | (0.018–0.036g) | (0.037–0.08g) | (0.003–0.05g) |
| Day 7                                | 120%    | 115%      | 41%                | 57%     | 67%       | 42%                | 134%    | 83%       | 18%                |
|                                      | (0.22g) | (0.40g)   | (0.1g)             | (0.007g)| (0.02g)   | (0.01g)            | (0.075g)| (0.12g)   | (0.03g)            |
|                                      | (0.12–0.32g) | (0.32–0.49g) | (0.04–0.2g)       | (0.003–0.01g) | (0.01–0.02g) | (0.004–0.02g) | (0.04–0.11g) | (0.083–0.15g) | (–0.003–0.06g) |
| Day 14                               | 175%    | 91%       | 26%                | 85%     | 35%       | 18%                | 153%    | 72%       | 13%                |
|                                      | (0.40g) | (0.45g)   | (0.06g)            | (0.007–0.02g) | (0.001–0.02g) | (–0.002–0.01g) | (0.073–0.16g) | (0.10–0.15g) | (–0.02–0.06g) |
|                                      | (0.29–0.51g) | (0.34–0.57g) | (0.03–0.1g)       | (0.007–0.02g) | (0.001–0.02g) | (–0.002–0.01g) | (0.073–0.16g) | (0.10–0.15g) | (–0.02–0.06g) |
| Day 28                               | 209%    | 162%      | 13%                | 97%     | 95%       | 4%                 | 223%    | 133%      | 16%                |
|                                      | (0.48g) | (0.69g)   | (0.04g)            | (0.005–0.03) | (0.02–0.04) | (–0.008–0.01g) | (0.098–0.21g) | (0.14–0.30g) | (–0.005–0.07g) |
|                                      | (0.55–0.61g) | (0.52–0.82g) | (–0.03–0.11g)     | (0.005–0.03) | (0.02–0.04) | (–0.008–0.01g) | (0.098–0.21g) | (0.14–0.30g) | (–0.005–0.07g) |

Numbers in parentheses are mean differences in absolute values and numbers in square brackets are 95% confidence limits.
although previous studies suggest that all intestinal layers are involved in the adaptive response (6, 11, 30). The present data clearly support the theory that the adaptive response is a transmural process, in proximal as well as distal small bowel. The significant adaptive hyperplasia of the muscularis propria layer observed at present and previously (30) deserves more attention, because it might have functional importance in relation to small bowel contractility and motility (4).

The most marked and inevitable effect of small bowel resection is the loss of absorptive area, which has prominent clinical consequences and in the most severe cases can lead to short bowel syndrome. It is well established that the adaptive response in some part compensates for the bowel loss by increasing the surface area of the remaining intestine. However, so far these important increases in surface area have not been quantified, probably because of a lack of proper methods. Modern stereological techniques offer a good solution to these problems, as demonstrated in this study.

The typical end points used in previous small bowel morphology studies have been villus height and cell counts along the crypt-villus axis. Besides the fact that these variables have limited value for interpreting the biological significance of changes in bowel morphology (23), another problem lies in the various sampling procedures used. Specimens are usually sampled from the bowel at one or two specific sites, which have not been defined before surgery. When the specimens are being examined microscopically, certain selection criteria are applied, e.g., the ten tallest villi or a specified number of perfectly sectioned crypts. As a result, the specimens may be neither representative nor comparable, and the fields of vision are not chosen randomly and independently of content and observer.

To our knowledge, the methods used in the present study and a number of previous experimental studies (19, 20, 22) represent the only existing practical solution to these problems. The marking out of small and large bowel segments during surgery provides standardized segments for later comparison and allows a reliable quantification of alterations in mass and surface area. Furthermore, the sampling requirements for vertical section stereology (2) secure a systematic and independent sampling and accordingly an unbiased quantification. We find the present unbiased stereo-
logical techniques highly recommendable for future experimental studies in this field.

It is generally assumed that the ileum displays a more robust adaptive response to resection than the jejunum (5, 35, 37) and that this should be explained by the unaccustomed spill of richer than normal chyme into the ileum and by relative increments in the luminal bulk received by the ileum in resected animals. Several studies using a short bowel model resembling the present one have indicated a greater adaptive potential of the ileum (11, 12, 21, 36), whereas we have only managed to find one study in which the data indicate the opposite (34). That study and the present one share in common the fact that similar food intake was ensured in the resected and sham-operated groups, in contrast to the studies cited supporting a greater adaptive potential of the ileum. It has been demonstrated that sham-operated rats are capable of eating greater amounts of food compared with resected animals (24, 26), implying that the jejunum of the sham-operated animals in this situation receives a greater luminal bulk of nutrients compared with the jejunum of the resected animals. Bearing in mind that luminal nutrition probably is the prime stimulus to intestinal growth (8, 21), it seems most likely that these essential differences in feeding regimens explain the conflicting results between the present and other studies.

However, it must be emphasized that, with the present knowledge, the general belief of a greater adaptive capacity of the ileum possibly holds true in short bowel models in which either an extensive proximal or distal resection (including the terminal ileum) has been performed (1, 6). It is also well recognized that in humans jejunal resections are often better tolerated than ileal ones. A contributory factor to the lack of jejunal adaptation in patients with ileectomy may be the removal of GLP-2-producing tissue (17). In contrast, elevated GLP-2 (and GLP-1) concentrations have recently been demonstrated in ileum-resected short bowel patients with the colon in continuity (18), which may contribute to the intestinal adaptation usually observed in these patients.

The contribution of endogenous GLP-2 to the development of intestinal hyperplasia has been studied in rodent models of experimental diabetes (9, 32), in which increased levels of circulating and intestinal GLP-2 were observed. These are correlative observations that suggest a link between intestinal growth and secretion of endogenous GLP-2. The present results show a similar, significant correlation between intestinal hyperplasia and an endogenous GLP-2 response and furthermore add new insight to the time-dependent changes of GLP-2 during the adaptive process in the rat short bowel model.

The most active adaptive growth took place during the first postoperative week, at which point we observed a significant peak in the circulating levels of GLP-2. This GLP-2 peak was surely not caused by an elevated food intake (17), because the intake was at a minimum during this period. At this early postoperative point we also detected a minor peak in GLP-2 levels among sham-operated rats. This indicates that the GLP-2 system is highly sensitive, and it might be speculated that the temporary response was caused by the laparotomy alone. For the last 2 wk of the study, the GLP-2 levels in the resected rats were continuously elevated to more than double the control values, and concurrently the remaining small bowel became additionally adapted. In this study, plasma concentrations of enteroglucagon (oxyntomodulin and glicentin) were elevated as well, in accordance with previous studies (3, 16). The concentrations of enteroglucagon were higher than those of GLP-2, possibly because of differences in the plasma elimination rates (31). But most importantly, the enteroglucagon levels were continuously elevated, paralleling the GLP-2 response, thus ensuring that the elevated GLP-2 levels observed were not just caused by substantially lower elimination rates. The receptor for GLP-2 has been characterized and cloned (25), and it was recently shown not to be directly expressed on proliferating crypt cells and villous enterocytes, pointing toward the existence of one or more downstream mediators of GLP-2 action (40).

The decreased circulating IGF-1 levels observed in the present study do not point toward a major role of circulating IGF-1 in the adaptive response but on the other hand do not rule out the possibility of IGF-1 acting as a local mediator of GLP-2 action. It must again be stressed that the present knowledge about GLP-2 and intestinal growth is correlative so far, and future studies using GLP-2 antagonists, immunoneutralizing GLP-2 antisera, or GLP-2 receptor knockout mice are warranted before the relative physiological importance of GLP-2 for intestinal adaptive growth after resections can be estimated.

The present study quantified the intestinal adaptive response after resection and demonstrated that all intestinal layers participated in the adaptive response. The adaptive response was most pronounced in the first week, and, in contrast to what is generally believed, the jejunum possessed the greatest adaptive capacity. The early adaptive response of the colon was temporary and gradually diminished as the remaining small bowel became fully adapted. The continuously elevated GLP-2 level in the resected rats is consistent with the hypothesis that GLP-2 plays an important role in the adaptive response after small bowel resection.

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